

Mirka Lumia

# Early life diet and asthma, with an emphasis on the role of fatty acids

## RESEARCH



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Mirka Lumia

# **Early life diet and asthma, with an emphasis on the role of fatty acids**

## **ACADEMIC DISSERTATION**

To be presented with the permission of the Board of the School of Health Sciences of the University of Tampere, for public examination at the small auditorium of Tampere School of Medicine B-building, Medisiinarinkatu 3, at noon on December 19 2014.

National Institute for Health and Welfare, Department of Lifestyle and Participation, Nutrition Unit

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**Supervisors**

Professor Suvi M. Virtanen  
Department of Lifestyle and Participation  
National Institute for Health and Welfare  
Helsinki, Finland

School of Health Sciences  
University of Tampere  
Tampere, Finland

Professor Minna Kaila  
Hjelt Institute  
University of Helsinki  
Helsinki, Finland

Department of Pediatrics  
Tampere University Hospital  
University of Tampere  
Tampere, Finland

**Reviewers**

Docent Sami Remes  
Department of Pediatrics  
Kuopio University Hospital  
Kuopio, Finland

Docent Kirsi Laitinen  
Functional Foods Forum  
University of Turku  
Turku, Finland

**Opponent**

Professor Päivi Rautava  
Department of Public Health  
University of Turku  
Turku, Finland

Turku Clinical Research Centre  
Turku University Hospital  
Turku, Finland



To my family



## Abstract

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Over the past 50 years, the prevalence of asthma and allergic diseases in childhood has increased, particularly in industrialized countries. In some countries, the prevalence is still rising, while in others the prevalence has reached a plateau or even decreased. In Finland, 7–9% of children have asthma and an equal number have asthma-like symptoms. Genetic predisposition cannot explain such a rapidly increased trend. Thus, it is suspected that the development of asthma requires multiple environmental exposures in genetically predisposed individuals. The role of nutrition during the perinatal period and infancy has been a focus of interest, because early life diet may modulate the development of the respiratory, digestive and immune system and it has been proposed that these changes persist until adulthood. Understanding the role of early life diet in the development of asthma would give us an opportunity to make dietary interventions already during the perinatal period and infancy.

The aim of the present study was to explore the associations between maternal diet during pregnancy and lactation, especially the role of fats and fatty acids (FA), as well as the child's diet in early life, including a milk-restricted diet with cow's milk allergy (CMA), on the risk of childhood asthma by the age of five years. We also investigated how the child's longitudinal serum FA proportions are associated with the risk of asthma.

This study was part of the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition Study, which began in 1996 at Oulu University Hospital and in 1997 at Tampere University Hospital. The genetic risk for type 1 diabetes may limit the generalizability of the results to the general population. Altogether 4065 children were invited to participate in the DIPP Allergy Study at the age of five years. All the required maternal dietary data and the child's asthma data were available for 2679 children in the pregnancy cohort and for 1789 children in the lactation cohort. Information on maternal diet during pregnancy and lactation was obtained retrospectively from the 181-item FFQ during the eighth month of pregnancy and third month of lactation. The nutrient variables were calculated as a percentage of energy to better describe the quality of the diet, not depending on the amounts consumed. The Cox proportional hazards regression was used in statistical analyses



to investigate the associations between maternal dietary intake during pregnancy and lactation and the risk of asthma in the offspring.

Information on asthma and at least one three-day food record were available for 182 children with asthma, and four matched controls were selected for each case. The amount of breast milk consumed was estimated based on growth and expected energy requirement. Information on asthma and at least one serum sample was available for 142 children with asthma, and two matched controls were selected for each case. Conditional logistic regression with a generalized estimating equation was used in statistical analyses to investigate the associations between longitudinal food consumption during early life, as well as repeated serum FA proportions, and the risk of later asthma.

During pregnancy, low maternal intakes of  $\alpha$ -linolenic acid (ALA, 18:3n-3) and total n-3 polyunsaturated fatty acids (PUFA) were associated with an increased risk of asthma in the offspring. By contrast, low maternal intakes of arachidonic acid (AA, 20:4n-6) and high maternal intakes of saturated fat (SAFA) and palmitic acid (16:0) were associated with a decreased risk of asthma. Maternal diet during lactation was not significantly associated with the risk of asthma in the offspring.

The diagnosis of CMA led to multiple dietary restrictions still evident at four years of age. Even after adjusting for CMA, higher consumption of cow's milk products (cow's milk-based infant formulas, fresh milk products, sour milk products, milk protein) was inversely associated with the risk of atopic asthma and higher consumption of breast milk and oats inversely associated with the risk of non-atopic asthma. Early consumption of fish was associated with a decreased risk of all asthma. CMA confounded the results and should be taken into account in further analysis.

The FA proportions of children with the diagnosis of CMA differed from those children without such a diagnosis. A higher serum proportion of eicosapentaenoic acid (EPA, 20:5n-3) was associated with a decreased risk of asthma after CMA was considered as a confounding factor. Higher proportions of linoleic acid (LA, 18:2n-6) and total n-6 PUFA were associated with an increased risk of atopic asthma. In addition, higher proportions of stearic acid (18:0) and total n-6 PUFA were associated with an increased risk of non-atopic asthma and higher proportions of EPA with a decreased risk.

The results from the present study provide evidence of the potential role of diet during the perinatal period and infancy on the risk of development of childhood asthma. CMA confounded the results of the associations between both childhood

diet and serum FA proportions and the risk of asthma. Changes in serum FA composition may predispose to CMA and subsequently to asthma because these diseases are on the same spectrum. Diagnosed CMA results in the elimination of milk and dairy products, and many other foods, from the diet, and thus induces other changes in addition to those found in serum FA profiles. These changes may modify the immunological reactions of the child already in early life and those changes may persist until adulthood.

Keywords: asthma, breast milk, cow's milk allergy, diet, epidemiology, fatty acids, lactation, pregnancy, serum fatty acids

## Tiivistelmä

Mirka Lumia. Varhainen ravitsemus ja astma. Terveiden ja hyvinvoinnin laitos. Tutkimus 145. 179 sivua. Helsinki, Finland 2014.

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Lapsuusiän allergisten sairauksien ja astman esiintyvyys on lisääntynyt viimeisten 50 vuoden aikana etenkin teollistuneissa maissa. Joissakin maissa esiintyvyys lisääntyy edelleen, osassa esiintyvyys on kääntynyt laskuun tai saavuttanut tasaisen vaiheen. Suomessa lapsista ja nuorista 7–9 % sairastaa lääkärin toteamaa astmaa ja vähintään yhtä monella on astman kaltaisia oireita. Astmaan liittyy voimakas perinnöllinen alttius. Perinnölliset tekijät muuttuvat kuitenkin hitaasti ja perinnöllinen alttius yksinomaan ei riitä selittämään astman esiintyvyyden nopeaa nousua. Nykytiedon mukaan astmaan sairastuminen vaatii perinnöllisen alttiuden lisäksi yhden tai useamman ympäristötekijän vaikutuksen, joista varhaisen ravitsemuksen on ajateltu olevan yksi merkittävä tekijä. Perinataaliajan ja lapsuusiän ravitsemus saattaa muokata hengitysteiden, ruoansulatuskanavan ja immuunijärjestelmän kypsymistä ja kehittymistä. Tällaiset muutokset voivat pysyä aikuisikään saakka. Varhaisen ravitsemuksen ja astman yhteyden selvittäminen antaisi mahdollisuuden ruokavaliomuutoksiin jo hyvin varhaisessa vaiheessa ja tällä saattaisi olla suuri kansanterveydellinen merkitys hoidettaessa astmariskissä olevia perheitä.

Väitöskirjatyön tarkoituksena oli selvittää äidin raskauden- ja imetyksen aikaisen ravitsemuksen sekä imeväis- ja lapsuusiän (5 vuoden ikään saakka) ruokavalion (sisältäen maidottoman ruokavalion lehmänmaitoallergisilla) yhteyttä lapsuusiän astman riskiin. Lisäksi tavoitteena oli selvittää voidaanko seerumin rasvahappoprofiilin eroilla (1–5 vuoden iässä) ennustaa lapsen tulevaa astmariskiä.

Tutkimus oli osa suomalaista tyypin 1 diabeteksen ennustamis- ja ehkäisy-tutkimuksen (DIPP) ravintotutkimusta. Tutkittu lapsiväestö koostuu tyypin 1 diabeteksen riskigeenin omaavista lapsista, joka saattaa rajoittaa tulosten yleistettävyyttä. DIPP ravintotutkimus alkoi vuonna 1996 Oulun ja 1997 Tampereen yliopistollisissa sairaaloissa. DIPP ravintotutkimuksen allergiaosioon kutsuttiin 4065 edelleen ravitsemusseurantaan kuuluvaa lasta 5 vuoden iässä. Tarvittava äidin ravinto- ja lapsen astmatieto oli käytettävissä 2679 lapsella raskauskohortissa ja 1798 lapsella imetyskohortissa. Astmatieto ja vähintään yksi kolmen päivän ruokapäiväkirja oli käytettävissä 182 lapsella. Jokaiselle astmatapaukselle valittiin neljä kaltaistettua verrokkia. Astmadiagnoosi ja vähintään yksi seeruminäyte oli käytettävissä 142 lapsella ja näistä jokaiselle valittiin kaksi kaltaistettua verrokkia. Tilastomenetelmänä käytettiin Coxin regressiota sekä logistista regressiota ja

yleistettyä estimointiyhtälöä (generalized estimating equations, GEE). Kulutetun rintamaidon määrä arvioitiin perustuen kasvuun ja oletettuun kasvuun tarvittavaan energiatarpeeseen. Lehmänmaitoaitoallergia oli tärkeä sekoittava tekijä tutkittaessa varhaisen ravitsemuksen ja astmariskin yhteyttä

Äidin raskaudenaikainen matala  $\alpha$ -linoleenihapon (18:3n-3) ja n-3 monityydyttymättömien rasvahappojen saanti oli yhteydessä lisääntyneeseen riskiin sairastua lapsuusiän astmaan. Matala arakidonihapon (20:4n-6) saanti puolestaan oli yhteydessä alhaisempaan astmariskiin. Korkea tyydyttyneiden rasvahappojen sekä palmitiinihapon (16:0) saanti oli yhteydessä pienempään astmariskiin. Äidin imetyksenaikainen ruokavalio ei ollut yhteydessä lapsen riskiin sairastua astmaan.

Lehmänmaitoallergia-diagnoosi johti merkittäviin ruokavaliorajoituksiin aina neljän vuoden ikään saakka. Korkeampi lehmänmaitotuotteiden (äidinmaidonkorvikkeet, tuoremaidotuotteet, hapanmaitotuotteet, maitoproteiini) käyttö lapsuusiässä oli käänteisessä yhteydessä atooppiseen eli IgE-positiiviseen astmaan. Korkeampi rintamaidon ja kauran käyttö vähensi riskiä ei-atooppiseen eli IgE-negatiiviseen astmaan. Varhainen kalan aloitus oli käänteisessä yhteydessä lapsuusiän astmariskiin.

Lehmänmaitoallergisten lasten seerumin rasvahappoprofiilit erosivat samanikäisistä ikätovereista ja välttämisruokavalio lisäsi näitä eroja entisestään. Lehmänmaitoallergiaa ei ole aiemmin huomioitu tutkittaessa seerumin rasvahappoprofiilin ja lapsuusiän astman yhteyttä. Korkeampi seerumin eikosapentaeenihapon (20:5n-3) osuus vähensi riskiä sairastua myöhempään astmaan, etenkin ei-atooppiseen astmaan. Korkeampi linolihapon (18:2n-6) sekä n-6 monityydyttymättömien rasvahappojen (n-6 PUFA) osuus lisäsi riskiä sairastua atooppiseen astmaan. Korkeampi steariinihapon (18:0) ja n-6 monityydyttymättömien rasvahappojen osuus lisäsi ei-atooppisen astman riskiä.

Tämän väitöskirjan tulokset puoltavat varhaisen ravitsemuksen vaikutusta lapsen astmariskiin. Jo äidin raskaudenaikainen ruokavalio vaikuttaa lapsen riskiin sairastua astmaan. Lehmänmaitoaitoallergia on tärkeä sekoittava tekijä tutkittaessa varhaisen ravitsemuksen ja astmariskin yhteyttä. Lehmänmaitoallergia-diagnoosi johti merkittäviin ruokavaliorajoituksiin aina 4 vuoden ikään saakka. Lehmänmaitoallergia ja astma kuuluvat samaan atooppiseen tautiperimään. Seerumin rasvahappoprofiilin muutokset sattaavat edeltää lehmänmaitoallergiaa ja astmaa. Diagnosoitu lehmänmaitoallergia johtaa välttämisruokavalioon ja sitä kautta edelleen suurempiin seerumin rasvahappoprofiilin muutoksiin. Nämä saattavat

muokata immuunipuolustusta jo hyvin varhaisessa vaiheessa. Tällaiset muutokset voivat olla pitkäkestoisia ja säilyä aikuisikään saakka.

Avainsanat: astma, rintamaito, lehmänmaitoallergia, ravitseminen, epidemiologia, rasvahapot, imetys, raskaus, seerumin rasvahapot

## Contents

Abstract	
Tiivistelmä	
Contents	
List of original papers	
Abbreviations	
1 Introduction	19
2 Literature review	21
2.1 Epidemiology of childhood asthma	21
2.1.1 Definitions, mechanisms and occurrence of asthma	21
2.1.2 Diagnosis and treatment of childhood asthma	22
2.1.3 Food allergies, asthma and hay fever	23
2.2 Factors associated with the development of asthma	25
2.2.1 Genetic factors	25
2.2.2 Environmental factors	25
2.2.3 Nutrition	26
2.3 Fatty acids and childhood asthma	27
2.3.1 Metabolism and the inflammatory and immunological effects of fatty acids	27
2.3.2 Dietary sources of fatty acids	33
2.3.3 Biomarkers for dietary fatty acids	33
2.3.4 The role of serum fatty acids in the development of atopic diseases (food allergy, hay fever or asthma)	34
2.4 Diet in early life	40
2.4.1 Diet during pregnancy, lactation and infancy during the study period in Finland: Recommendations and actual diet	40
2.4.2 The role of early life diet in the development of asthma and atopic diseases	42
2.5 Summary of the literature review	67
3 Aims of the study	68
4 Materials and methods	69
4.1 Subjects and study design	69
4.2 Outcome assessment	72
4.2.1 Asthma diagnosis (Study I-IV)	72
4.2.2 Immunoglobulin E (IgE) measurements	73
4.2.3 Food allergy diagnosis	73
4.3 Dietary methods	73
4.3.1 Food frequency questionnaire	73
4.3.2 Three-day food record	74
4.3.3 Age-specific dietary questionnaires	75

4.3.4 Estimation of breast milk intake .....	75
4.4 Serum fatty acids .....	75
4.5 Sociodemographic and other background characteristics of the study population .....	76
4.6 Statistical methods .....	76
4.6.1 Logistic regression and cox proportional hazards regression (Study I and II) .....	76
4.6.2 Generalized estimating equation framework, conditional logistic regression (Study III and IV) .....	77
4.7 Ethical issues .....	79
5 Results .....	80
5.1 Characteristics of the study population .....	80
5.2 Maternal fat and fatty acid intake during pregnancy and lactation and the risk of asthma in the offspring by the age of five years (Study I and II) .....	80
5.3 Food consumption during infancy and childhood and the risk of asthma (Study III) .....	87
5.4 Serum fatty acids and the risk of asthma (Study IV) .....	90
6 Discussion .....	91
6.1 Summary of the findings .....	91
6.2 Strengths and limitations of the study .....	92
6.2.1 Study design and endpoints .....	92
6.2.2 Subjects and generalizability .....	93
6.2.3 Food frequency questionnaire, three-day food records and serum samples .....	93
6.3 Comparison of results with earlier studies .....	95
7 Conclusions and future implications .....	100
8 Acknowledgements .....	101
Appendix .....	104
References .....	106

## List of original papers

- I Mirka Lumia, Päivi Luukkainen, Heli Tapanainen, Minna Kaila, Maijaliisa Erkkola, Liisa Uusitalo, Sari Niinistö, Michael G. Kenward, Jorma Ilonen, Olli Simell, Mikael Knip, Riitta Veijola, Suvi M. Virtanen. Dietary fatty acid composition during pregnancy and the risk of asthma in the offspring. *Pediatr Allergy Immunol*. 2011;22:827-35.
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## Abbreviations

CI	confidence interval
CMA	cow's milk allergy
DBPCFC	double-blind, placebo-controlled food challenge
DIPP	Type 1 Diabetes Prediction and Prevention Study
FA	fatty acids
AA	arachidonic acid (20:4n-6)
ALA	$\alpha$ -linolenic acid (18:3n-3)
CLA	conjugated linolenic acid (18:2n-6, con)
DGLA	dihomo- $\gamma$ -linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (22:6n-3)
DPA	docosapentaenoic acid (22:5n-3)
EPA	eicosapentaenoic acid (20:5n-3)
GLA	$\gamma$ -linolenic acid (18:3n-6)
LA	linoleic acid (18:2n-6)
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acids
SAFA	saturated fatty acids
FADS	fatty acid desaturase
FAS	fatty acid synthetase
FEV <sub>1</sub>	forced expiratory volume during the first second of forced expiration
FFQ	food frequency questionnaire
FVC	forced expiratory vital capacity

GEE	generalized estimating equation
HLA	human leucocyte antigen
HR	hazard ratio
IgE	immunoglobulin E
ISAAC	International Study of Asthma and Allergies in Childhood
OR	odds ratio
PEF	peak expiratory flow
PGE2	prostaglandin E2
SD	standard deviation
SE	standard error
Th1	T-helper 1 lymphocyte
Th2	T-helper 2 lymphocyte



# 1 Introduction

Asthma and allergic diseases are among the most common chronic diseases of childhood (Asthma: Current Care Guidelines Abstract 2013). The prevalence of asthma and allergic diseases has almost doubled in westernized countries in the last three decades (Mickleborough *et al.* 2013). The incidence of asthma has continued to increase in developing countries, while it has stopped or even decreased in developed countries (von Herzen *et al.* 2005, Pearce *et al.* 2006). The incidence of childhood asthma has shown no plateau, although large variations in the prevalence of childhood asthma have been described in different districts. In Finland the prevalence of childhood asthma among school-aged children is 7–9% and an equal number have asthma-like symptoms (Hugg *et al.* 2008, Lai *et al.* 2009, Asthma: Current Care Guidelines Abstract 2013). The number of children receiving special reimbursement for asthma medication in Finland was 1.9 per 100 children in 1994, 3.1 per 100 children in 2002, and 2.6 per 100 children in 2006 (Mäkelä *et al.* 2008). Globally, among children aged six to seven years, the total prevalence of asthma was 11.5%, ranging from 3.4% in Africa and 29.2% in Oceania (Lai *et al.* 2009).

Although asthma and allergic diseases have a strong genetic predisposition, genetic factors cannot explain such rapid changes in the incidence of asthma (Global Initiative for Asthma (GINA) 2014). Thus, the incidence of asthma and allergic diseases is determined both by genetic and environmental factors. Environmental factors, such as dietary and microbial factors may interact, resulting in different asthma phenotypes. In addition, exposure to tobacco smoke, air pollutants and allergens have been linked to asthma (GINA 2014). The original hygiene hypothesis suggests that the increased incidence of asthma and allergic diseases is linked to diminished microbial exposure during early childhood, cleanliness and the reduced number of children in families (Strachan 1989, Strachan 2000, Brooks *et al.* 2013). Furthermore, there is evidence that diet in early life, even already *in utero*, may modulate the development of the airways, the digestive system and gut microbiota, and the immune system. This development of these organ systems begins already *in utero* (Devereux *et al.* 2002).

During urbanization, our diet changed from traditional foods towards more processed foods. Foods are also stored for longer periods and transported over long distances (Devereux 2007). It has been proposed that the increase in the asthma burden in line with westernization may partly be related to a higher intake of n-6 PUFA found in margarines and vegetable oils, and a lower intake of n-3 PUFA found in marine oils (Black *et al.* 1997). However, the changes are not similar in all

countries. N-3 PUFA have anti-inflammatory properties, while n-6 PUFA, especially arachidonic acid (AA), are precursors of pro-inflammatory eicosanoids, such as prostaglandins, through AA-derived eicosanoids (Prescott *et al.* 2004). Prostaglandin E2 (PGE2) alters the balance between Th1 and Th2 cytokines toward Th2 dominance, thus enhancing immunoglobulin E (IgE) production and the development of IgE-mediated allergic diseases such as asthma (Wood *et al.* 2009).

Diet is a flexible exposure and discovering the role of early life diet in the development of asthma would give us an opportunity to perform well-designed randomized controlled trials or real life experiments on dietary interventions already during the perinatal period and infancy (Devereux 2007).

The aim of the present study was to assess the role of the early life nutrition (including milk free diet in children with cow's milk allergy (CMA)), especially the intake of fats and fatty acids, in the development of childhood asthma by the age of five years.

## 2 Literature review

### 2.1 Epidemiology of childhood asthma

#### 2.1.1 Definitions, mechanisms and occurrence of asthma

Childhood asthma is defined as a chronic inflammatory disease of the respiratory tract, which is characterized by a reversible airway obstruction, inflammation and bronchial hyperreactivity resulting in recurrent episodes of wheezing, breathlessness chest tightness and coughing. Both the adaptive and the innate immunity are involved in the pathogenesis of asthma. The presence of T helper type 2 (Th2) lymphocytes, eosinophils and basophils are typical for asthmatic reaction type. This reaction type leads to increased mucus production, mucosal edema, reversible airway obstruction, bronchial hyper-responsiveness and airway remodeling. IgE-positive asthma is defined as atopic asthma and IgE-negative asthma as non-atopic asthma. (Global Initiative for Asthma (GINA) 2014, Papadopoulos *et al.* 2012)

Until recently, asthma was believed to be an atopic disease caused by exposure to allergens. Nowadays, it is thought to be a complex syndrome, and it is known that about half of asthma cases are related to atopy, while the other half are non-atopic (Pearce *et al.* 2012). There is growing evidence that childhood asthma consists of several disease entities, different phenotypes, and varying prognoses (Martinez *et al.* 1995, Brand *et al.* 2008, Weinmayr *et al.* 2013). Asthma symptoms may be intermittent and non-specific and it is sometimes difficult to set the correct diagnosis for asthma. Early wheezing symptoms should be defined as episodic (wheezing associated with viral infections) or a multiple-trigger wheeze (Brand *et al.* 2008). Children with episodic wheezing are asymptomatic between the viral infections but multiple-trigger wheezers also wheeze in response to stimuli other than viral infections (Kalliola *et al.* 2013).

An increasing trend of asthma and atopic diseases has been observed over the past fifty years in westernized countries (Haahtela *et al.* 2012). However, the International Study of Asthma and Allergic diseases in Childhood (ISAAC) Phase III reported that asthma prevalence has reached a peak or even decreased in adults in Western countries (von Hertzen *et al.* 2005, Pearce *et al.* 2006, Pearce *et al.* 2012). The incidence of childhood asthma has shown no plateau, although large variations

in the prevalence of childhood asthma have been described in different districts. Lower incidence has been found in some rural areas of Africa, in Asia, and among farmer's children in Europe (Pearce *et al.* 2006, Lai *et al.* 2009).

Asthma is a worldwide problem affecting about 300 million individuals (GINA 2014). The prevalence of asthma ranges from 1% to 18% of the population in different countries (GINA 2014). Globally, the prevalence for current asthma in the 13–14-year age group was 14.1%, and in the 6–7-year age group 11.7% (Mallol *et al.* 2013). In Finland, 7–9% of children have a diagnosis of asthma and an equal number have asthma-like symptoms (Hugg *et al.* 2008, Lai *et al.* 2009, Asthma: Current Care Guidelines Abstract 2013). In a retrospective study of childhood asthma in Finland between 1976 and 1995, hospital admissions have increased 2.8-fold, but the length of hospital stay has halved (Malmström *et al.* 2000). It seems that the prevalence of mild and moderate asthma has increased, while the prevalence of severe asthma has remained unchanged (Malmström *et al.* 2000). The number of children receiving special reimbursement for asthma medication in Finland was 1.9 per 100 children in 1994, 3.1 per 100 children in 2002, and 2.6 per 100 children in 2006 (Mäkelä *et al.* 2008).

### 2.1.2 Diagnosis and treatment of childhood asthma

The diagnosis of asthma is based on clinical symptoms, the demonstration of reversible airway obstruction in different lung function tests, a medication trial, and the exclusion of other diseases causing same symptoms (GINA 2014, Asthma: Current Care Guidelines Abstract 2012). Clinical symptoms include cough, mucus production, shortness of breath and wheezing. The diagnosis is confirmed with agreements of values one in one of the following lung function tests:

1. Peak expiratory flow (PEF) improvement of 15% or more of the pre-bronchodilator PEF at least three times, or 20% or more of the diurnal variation in PEF during a 2-week study period;
2. Forced expiratory volume (FEV<sub>1</sub>) or forced expiratory vital capacity (FVC) improvement of more than 12% or at least 200 ml after bronchodilator in spirometry;
3. 15% or more fall in histamine or metacholine tests;
4. 15% or more fall in FEV<sub>1</sub> after a run exercise test;
5. 40% increase in resistance with a bronchodilator test or a 40% fall with a run exercise test in oscillometry.

Small children often have wheezing associated with viral infections. Oscillometry and run exercise tests are usually successfully performed in children over three years of age and spirometry with a bronchodilatory test in children over five years of age.

The exhaled nitric oxide test reflects the eosinophilic inflammation of the airways and is suitable for preschoolers and school-aged children (GINA 2014).

In children under three years of age, the diagnosis is based on clinical symptoms. If the child has obstruction at least three times per year and has in addition risk factors for asthma (Table 1), a medication trial can be started (Asthma: Current Care Guidelines Abstract 2012).

**Table 1.** Risk factors for asthma in children under three years old who have recurrent wheezing symptoms. Children having at least one main criterion or two side criteria are at risk for asthma (Castro-Rodriguez *et al.* 2000, Castro-Rodriguez 2010).

Main criteria	Side criteria
1. Doctor-diagnosed asthma in one of the parents	1. IgE-associated sensitization to foods
2. Doctor-diagnosed atopic eczema	2. Wheezing without viral infections
3. Sensitization to inhalant allergens	3. Blood eosinophils >4% or $>0.4 \times 10^9/l$

If the medication is needed for six months or more, the asthma diagnosis is set. Allergic (IgE-mediated) and non-allergic asthma should be distinguished (GINA 2014).

The goal of the treatment of asthma is normal lung function without asthma symptoms and the prevention of the exacerbation of asthma. In most cases, good symptom control can be achieved with inhaled corticosteroids. Short-acting  $\beta_2$ -agonists can be used if needed. Long-acting  $\beta_2$ -agonists in fixed combination with inhaled corticosteroids and a leukotriene receptor antagonist may be added to the anti-asthmatic medication in more severe asthma (GINA 2014).

### 2.1.3 Food allergies, asthma and hay fever

Asthma and allergic diseases are common diseases with complex and heterogeneous etiologies. They often co-occur within the same individual or in the same family. The hypothesis of “The Allergic March” describes the progression of atopic eczema and food allergy in infancy to asthma and hay fever in childhood. The natural course of hypersensitivity changes from foods to inhalant allergens (Kjaer *et al.* 2009).

According to a Danish cohort study, early sensitization to cow’s milk and egg, as well as early atopic eczema, are strong predictors for later allergic diseases and



asthma (Kjaer *et al.* 2009). Egg and tree nut allergies have been shown to be associated with later childhood asthma (Gaffin *et al.* 2011). By contrast, children sensitized early transiently to inhalant allergens did not have an increased risk for later allergic disease (Kjaer *et al.* 2009).

Three to eight percent of the children have food allergies (Leung *et al.* 2014). In addition to genetic factors, epigenetic alterations and environmental factors are associated with the development of food allergy (Hong *et al.* 2009). Male sex, Asian and black ethnicity, atopy, low vitamin D levels, low intakes of n-3 PUFA, obesity, increased hygiene and the timing of introduction of solid foods are proposed to be risk factors for food allergies (Leung *et al.* 2014). Cow's milk and egg allergies are the most common food allergies among children. Two to three percent of children have CMA and 1–2% have an egg allergy (Leung *et al.* 2014). In Finland, 1.9% of children under twelve months of age have challenge-confirmed CMA (Saarinen *et al.* 1999). Childhood allergies to cow's milk, egg, wheat and soy usually elucidate among preschoolers, while allergies to peanut, tree nut, fish and shellfish usually persist (Leung *et al.* 2014).

CMA is an adverse immunologic reaction to the ingested cow's milk protein. CMA can be IgE-mediated, non-IgE-mediated or a mixture of both (Venter *et al.* 2011). Typical symptoms of IgE-mediated CMA usually occur rapidly after ingestion of cow's milk and consist of skin symptoms (urticaria, erythema, angioedema), respiratory symptoms (wheeze, cough, stridor, nasal congestion, respiratory distress) and anaphylaxis (Venter *et al.* 2011). In non-IgE-mediated (cell-mediated) CMA, symptoms usually come from the gastrointestinal tract (vomiting, diarrhea, abdominal pain) and have later onset compared with IgE-mediated CMA (World Allergy Organization 2010, Merras-Salmio *et al.* 2013, Merras-Salmio *et al.* 2014). The diagnosis of CMA arises from clinical symptoms and physical examination, and is confirmed by the presence of cow's milk-specific IgE (skin prick test or immunoassay of serum antigen specific IgE) and a positive oral food challenge (Longo *et al.* 2013). A double-blind, placebo-controlled food challenge (DBPCFC) is the gold standard for diagnosis (Longo *et al.* 2013, Merras-Salmio *et al.* 2013). However, DBPCFC is time-consuming and laborious, and open or single-blind food challenges are often used (Longo *et al.* 2013). In epidemiological studies, the diagnosis is often based on parental reports and it thus may be overdiagnosed (Venter *et al.* 2011).

## 2.2 Factors associated with the development of asthma

Factors that influence the development and expression of asthma can be divided into those that are associated with the development of asthma (host factors: genetic, obesity, sex) and those that trigger asthma symptoms (environmental: allergens, infections, tobacco smoke, air pollution, diet) (GINA 2014). However, the mechanisms are complex and interactive, and there is an overlap between these factors. Asthma usually develops in subjects with a genetic susceptibility after environmental stimulus. This phenomenon is called gene-environment interaction (GINA 2014).

### 2.2.1 Genetic factors

Family history and twin studies have shown that genetics play an important role in the development of asthma (Subbarao *et al.* 2009). The most important risk factor for the development of asthma is the same disease in parents or siblings (Subbarao *et al.* 2009). Multiple genes may be involved in the pathogenesis of asthma and nearly 100 genes have recently been identified as being related to asthma and asthma-related phenotypes (Duijts *et al.* 2012). There are replicated regions on the long arms of chromosomes 2, 5, 6, 12 and 13 associated with asthma (Subbarao *et al.* 2009). In genome-wide association studies, a locus on chromosome 17q12–21 has been identified as a risk factor for childhood-onset asthma (Dijk *et al.* 2013). Many genes with small effects instead of a few genes with major effects may be responsible for the development of asthma (Subbarao *et al.* 2009). There are genes affecting atopy and genes affecting to airway hyperresponsiveness (GINA 2014). In patients with allergic rhinitis, the risk for asthma is 2–4-fold higher compared with the general population (GINA 2014). Children at genetic risk for later developing asthma are suspected to be born with impaired lung function, and the lung function further decreases when the child grows older (von Mutius 2012). In addition to genes that predispose to asthma, there are also genes associated with the response to asthma treatment with  $\beta_2$ -agonists and glucocorticoids (GINA 2014).

### 2.2.2 Environmental factors

The increased incidence of asthma has been explained by the westernized style of living (Devereux 2007). This lifestyle includes increased air pollution, reduced microbial exposures, higher use of antibiotics, changes in dietary habits in mothers during pregnancy and lactation and in children, short duration of breastfeeding, smaller family size, and a lower exposure to farming environment and pets (von Hertzen *et al.* 2005). These changes lead to inadequately developed tolerance, inappropriate inflammatory responses and clinical symptoms of asthma (Haahtela *et*

*al.* 2012). Tobacco smoke during pregnancy and passive smoking postnatally are well-known etiological factors in childhood asthma (Bisgaard *et al.* 2012).

### 2.2.3 Nutrition

The concept of "early life programming" defines the role of environmental exposures during the prenatal period on the production of both structural and functional effects on the immune system that persist even into adulthood (Barker 1998, Barker 1999, Calder 2010). Epigenetic mechanisms are recently proposed to play a key role in this process by which dietary exposures can lead to changes in immune development (Amarasekera *et al.* 2013). Epigenetic mechanisms are defined as a network of biological processes that regulate the expression of genes, to produce changes in cellular function without changes in underlying DNA-sequence (Amarasekera *et al.* 2013).

Early life is characterized by a rapid growth and development. Inadequate diet may restrict growth and the full utilization of the genetic potential of the infant (Hermoso *et al.* 2010). Adequate nutrition is critical in early life to support normal growth and development. The early life diet is the focus of interest in asthma because airways and the digestive and immune systems are developing during that time period (Hageman *et al.* 2012). The evidence does not support the associations between dietary interventions as a treatment in already established asthma in children and adults (Thien *et al.* 2002, Mickleborough *et al.* 2013). Because atopy may mostly be programmed in early life, dietary interventions may be too late once the allergic process has already started (Calder 2010).

Breast milk contains immunomodulatory components and exclusive breastfeeding for at least four months has been proposed to protect children from asthma (Kneepkens *et al.* 2010). Formula-fed infants have higher incidence of wheezing compared to breastfed infants (GINA 2014). The role of introduction of solid foods to the diet has changed during recent years. The delayed introduction is not longer recommended for prevention of allergic diseases (Fleischer *et al.* 2012). There is some evidence of the association of introduction of complementary feeding between four and six months of age with a decreased risk of asthma and allergic diseases (Fleischer *et al.* 2012, Nwaru *et al.* 2013).

Dietary hypotheses have mainly focused on PUFA, vitamin D, antioxidants, Mediterranean diet, and fruit, vegetable and fish consumption (Chatzi *et al.* 2009, Devereux *et al.* 2010, Antó 2012, Torres-Borrego *et al.* 2012, Saadeh *et al.* 2013). Increased use of processed foods, foods rich in n-6 PUFA and decreased use of n-3 PUFA and antioxidant-containing foods and vitamin D have been associated with an

increased risk of asthma (Devereux *et al.* 2010, West *et al.* 2010). Higher consumption of n-3 PUFA and antioxidant containing foods during early life has been inversely associated with childhood asthma (Devereux *et al.* 2010, West *et al.* 2010). Frequent consumption of fruits, vegetables and fish during childhood was associated with a lower lifetime prevalence of asthma in ISAAC Phase II study (Nagel *et al.* 2010). However, higher intake of fish oil and vitamin D during infancy has failed to show protective effects against childhood asthma (Kremmyda *et al.* 2011). Higher maternal intake of antioxidant-containing foods, fish and vitamin D has been associated with a decreased risk of asthma in the offspring (West *et al.* 2010).

## 2.3 Fatty acids and childhood asthma

### 2.3.1 Metabolism and the inflammatory and immunological effects of fatty acids

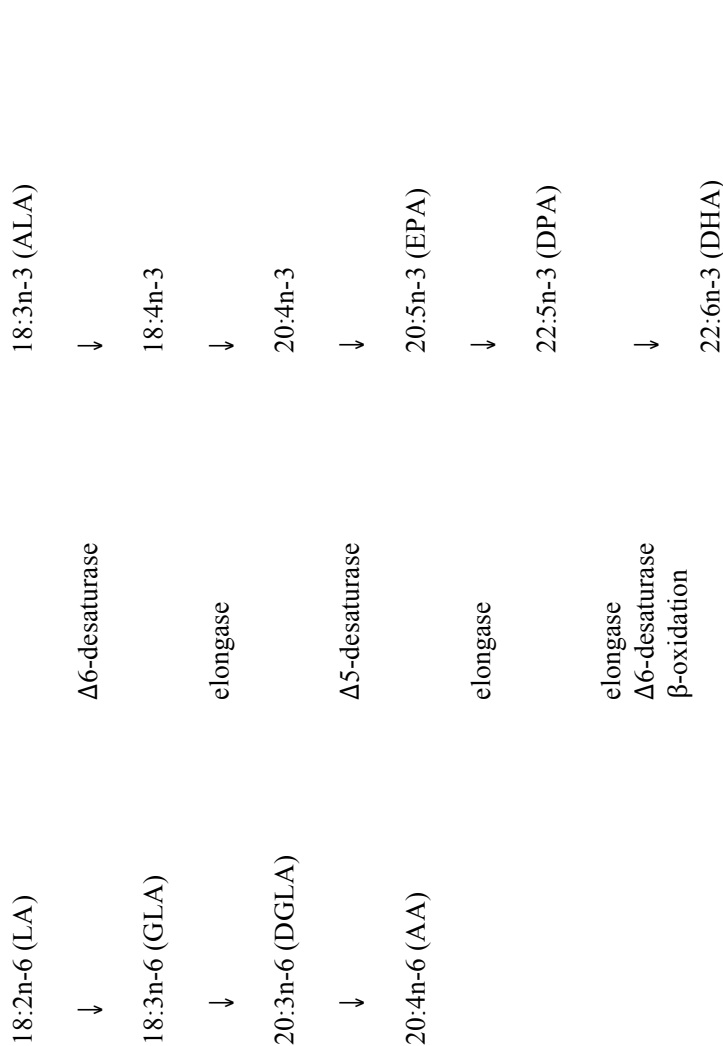
FA of animal origin are usually straight-chain derivatives containing an even number of carbon atoms. The chain may be saturated (containing no double bonds) or unsaturated (containing one or more double bonds). FA containing one double bond in the acyl chain are called monounsaturated FA, while FA containing two or more double bonds are called PUFA.

FA are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other (Murray *et al.* 2003). FA are numbered by their carbon chain length which varies from two to thirty or more carbon atoms. The numbering system for FA shows the number of carbon atoms and double bonds, and the position of the first double bond from the end of the carbon chain opposite the carboxyl group (Murray *et al.* 2003). Carbon atoms are numbered starting from the carboxyl or  $\Delta$ -terminus. The carbon atoms adjacent to the carboxyl carbon (2, 3 and 4) are also known as the  $\alpha$ ,  $\beta$  and  $\gamma$  carbons, respectively. The terminal methyl carbon is known as the  $\omega$  or n-carbon. Usually  $\Delta$  is used for indicating the number and position of the double bonds. For example,  $\Delta^3$  indicates a double bond between carbons three and four of the FA,  $\omega 3$  indicates a double bond on the third carbon counting from the  $\omega$ -carbon. If the acyl chains are on the same side of the bond, it is *cis*-, if in opposite sides, it is *trans*-isomer. Naturally occurring unsaturated long-chain FA are nearly all of the *cis* configuration (Murray *et al.* 2003).

There are three families of PUFA, the n-9, n-6 and n-3 families. Oleic acid (18:2n-9) is the starting point of synthesis of the n-9 FA and can be endogenously synthesized. The simplest members of the n-6 and n-3 families, linoleic acid (18:2n-

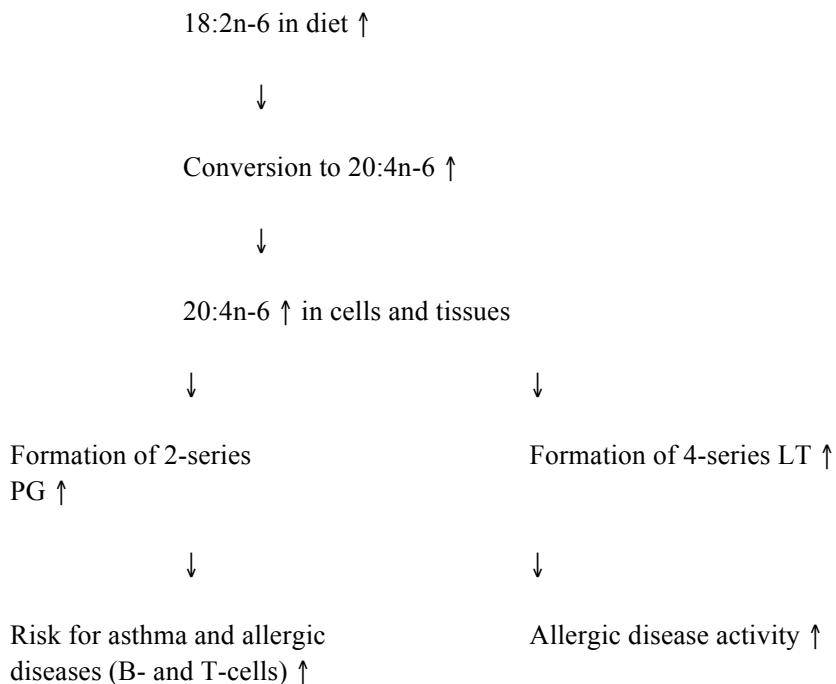
6, LA) and  $\alpha$ -linolenic acid (18:3n-3, ALA) cannot be synthesized by mammals and must be obtained from the diet, so are called essential FA (Wall *et al.* 2010).

PUFA constitute an important component of cell membranes and influence membrane fluidity and the behavior of membrane-bound enzymes and receptors (Sala-Vila *et al.* 2008). The liver is the major site for the metabolism of PUFA, both for synthesis of hepatic membrane phospholipids and for export and uptake by most other cells. Long chain PUFA are synthesized from the nutritionally essential FA, LA of the n-6 series and ALA of the n-3 series (Kremmyda *et al.* 2011). Long chain PUFA are produced from these FA in tissues by chain elongation and desaturation procedures. LA is converted to GLA by the action of the enzyme  $\Delta^6$ -desaturase. GLA is further elongated to dihomo-GLA (DGLA), which is a precursor of 1 series prostaglandins (PG). DGLA can also be converted to AA by the action of  $\Delta^5$ -desaturase (Sala-Vila *et al.* 2008). Figure 1 shows the biosynthesis of FA.



**Figure 1.** Biosynthesis and metabolism of n-6 and n-3 PUFA (modified from Murray *et al.* 2003, Kremmyda *et al.* 2011).  
AA=arachidonic acid, ALA= $\alpha$ -linoleic acid, DGLA=dihomo- $\gamma$ -linolenic acid, DHA=docosahexaenoic acid, DPA=docosapentaenoic acid, EPA=eicosapentaenoic acid, GLA= $\gamma$ -linolenic acid, LA=linolenic acid, PUFA=polyunsaturated fatty acids.

In addition to their role as the essential components of membrane structures and their functional properties, PUFA also have an important role in regulating inflammatory responses through production of inflammatory mediators termed eicosanoids: prostaglandins (PG), thromboxanes and leukotrienes (LT). These biologically active mediators have inflammatory properties and are involved in various disease processes, like asthma and atopic diseases (Figure 2) (Calder *et al.* 2009).



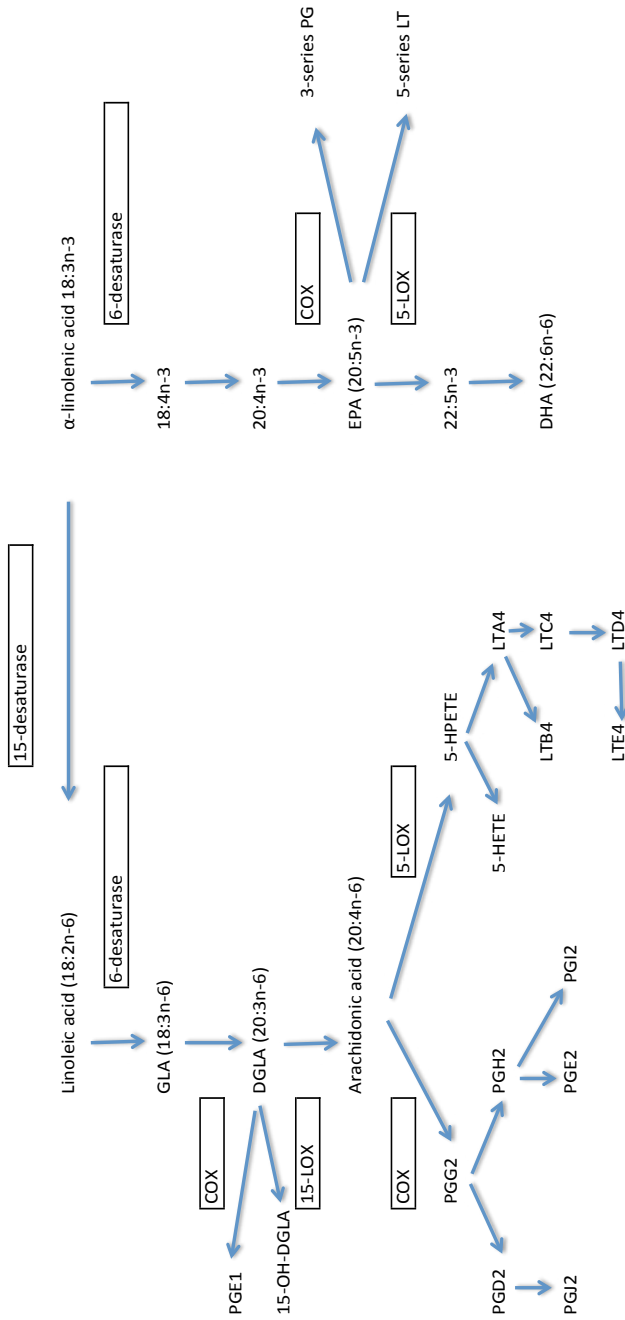
**Figure 2.** Mechanism between increased n-6 PUFA exposure and atopic diseases (modified from Kremmyda *et al.* 2011).

LT=leukotrienes, PG=prostaglandins, PUFA=polyunsaturated fatty acids.

Figure 3 shows the metabolism of n-3 and n-6 PUFA. Eicosanoids are key mediators and regulators of inflammation and are generated from PUFA. AA is a precursor of pro-inflammatory eicosanoid mediators that have established roles in inflammation, like 2 series PG, thromboxanes and 4-series LT. However, it is recently shown that PGE<sub>2</sub> have both pro- and anti-inflammatory properties thus indicating that some AA-derived eicosanoids may be important in turning off inflammation (Calder *et al.* 2009).

ALA is converted to EPA by the action of enzymes  $\Delta^5$ - and  $\Delta^6$ -desaturase (Kremmyda *et al.* 2011). EPA can be further converted to DHA. Oily fish and fish oils contain EPA and DHA. Increased consumption of these FA results in their incorporation into inflammatory cell phospholipids in a time- and dose-dependent manner (Kremmyda *et al.* 2011). EPA and DHA inhibit AA metabolism. EPA is also a precursor for the less potent anti-inflammatory 3-series PG and 5-series LT (Prescott *et al.* 2004). Fish oil supplementation has been shown to result in increased production of LTB<sub>5</sub>, LTE<sub>5</sub> and 5-hydroxyeicosapentaenoic acid by inflammatory cells (Calder *et al.* 2009). However, it was recently shown that EPA and AA-derived eicosanoids may also have the same kind of potencies (Calder *et al.* 2009). EPA is also a precursor for E-series resolvins and DHA for D-series resolvins and protectins that appear to have anti-inflammatory and inflammation-resolving actions. Many of the effects of n-3 PUFA on inflammatory mediator production may be related to the altered expression of the genes coding those mediators rather than to changes in eicosanoid production. There are also observations of a low AA status in atopic diseases. Therefore, it may be ideal to combine fish oil with some long chain n-6 PUFA to prevent asthma and allergic diseases (Kremmyda *et al.* 2011).





**Figure 3.** Metabolism of n-6 and n-3 PUFA (modified from Calder *et al.* 2009).

### 2.3.2 Dietary sources of fatty acids

ALA and LA are synthesized by plants and are found in plant tissues, such as leaves, nuts, seeds and seed oils (Miles *et al.* 2014, Wendell *et al.* 2014). The main dietary sources of LA are cereals, eggs, poultry, peanut, vegetable oils (corn, sunflower and soybean oils), whole-grain breads and margarines (Murray *et al.* 2003, Das 2006). ALA is found in green plant tissues and the dietary sources of ALA are canola oil, flaxseed oil, linseed and rapeseed oils, walnuts and green leafy vegetables (Das 2006). Olive oil is rich in oleic acid. AA is mostly obtained from meat, egg yolks, some seaweeds, shrimps and peanut oil. Fish and fish products are the main sources of EPA and DHA (Das 2006, Miles *et al.* 2014, Wendell *et al.* 2014).

Breast milk is rich in GLA (0.3–1%) compared with cow's milk (0.25%) (Koletzko *et al.* 2008). Breast milk contains moderate amounts of AA whereas cow's milk contains only small amounts. Human milk provides LA, ALA, DHA, AA and other LC-PUFAs to breastfed infants (Koletzko *et al.* 2008). The level of AA is relatively constant whereas the level of DHA varies depending on maternal diet and lifestyle factors (Koletzko *et al.* 2008). Infant formulas are prepared to match the FA composition of breast milk. However, the circulating levels of AA and DHA in formula-fed infants are lower compared with breastfed infants unless DHA and AA are supplemented (Koletzko *et al.* 2008).

The main sources of fats among Finnish preschoolers at the age of one year were milk containing one or 1.5% fat, yoghurt and meat dishes. Most of the fat from cereal products at the age of one year came from porridge, while salty and sweet pastries and biscuits were the usual sources of them at the ages of three and six years. In addition, cheese and milk desserts, fat spreads and meat dishes are common sources of fat (Kyttälä *et al.* 2010).

According to the National Findiet 2012 Survey, among the working aged women, most of the SAFA consumed was so-called hidden fat, derived from milk products, meat products and bakery products. The proportion of fat and SAFA was higher compared to the National Findiet 2007 Survey (Helldan *et al.* 2012). The most important sources of essential FA were vegetable oil-based spreads and oils. Margarines were the most important source of essential FA (Helldan *et al.* 2012).

### 2.3.3 Biomarkers for dietary fatty acids

Specific FA levels in blood, cell membranes and subcutaneous fat are indicators of dietary fat intake (Willet 1998). Individual FA levels can be measured in erythrocytes, platelets, adipose tissue and in several lipid fractions found in plasma

(Willett 1998). Serum cholesteryl ester is the most suitable serum fraction to measure short-term dietary intake, while adipose tissue is the biomarker for long-term dietary intake (Baylin *et al.* 2006). Serum FA may be measured in the cholesteryl ester, phospholipid and triglyceride fractions of serum or plasma or as free FA (Willett 1998, Baylin *et al.* 2006). Since the correlation between PUFA in diet and in tissues may be weakened by desaturation, elongation and other metabolic processes, the best markers of dietary intake are FA that cannot be endogenously synthesized from carbohydrates (Willett 1998). N-6 and n-3 PUFA, trans FA and odd-numbered and branched-chain SAFA are proposed as the most optimal biomarkers among FA (Baylin *et al.* 2006). Serum and plasma FA represent transport pools in the movement of FA between body components. This includes the transportation of FA to immune cells (Baylin *et al.* 2006).

Serum and plasma FA composition is influenced by diet and by metabolic processes. It has been suggested that the atopic disease process itself may also affect serum FA composition. A difference in the FA composition of serum between atopic and non-atopic individuals would indicate a different exposure of body compartments to FA, and may reflect diet, metabolism or the disease process (Sala-Vila *et al.* 2008).

#### **2.3.4 The role of serum fatty acids in the development of atopic diseases (food allergy, hay fever or asthma)**

Studies have compared the serum FA composition of atopic and non-atopic children and also cord blood FA composition between children who later became atopic and those who did not (Sala-Vila *et al.* 2008). Cord blood lipids from neonates who developed atopic disease seems to contain lower proportions of DGLA, AA and perhaps EPA and DHA, and also a higher proportion of LA compared with children without atopic disease (Sala-Vila *et al.* 2008). In addition, serum obtained in early infancy seems to contain lower amounts of DGLA, GLA and AA in children who developed atopic disease. Higher LA status has not been shown in atopic individuals, neither is there clear evidence of the role of serum n-3 PUFA in atopic diseases. However, some studies have reported lower amounts of EPA and DHA (Sala-Vila *et al.* 2008).

Taken together, these data do not support the lipid hypothesis induced by Black and Sharpe (Black *et al.* 1999). It has been suggested that these lower levels of n-6 PUFA may represent an insufficient ability to convert LA into longer-chain metabolites in atopic children, or it may represent the increased usage of these FA in atopic children (Sala-Vila *et al.* 2008). Table 2 summarizes the earlier findings between serum FA and asthma.

Table 2.

Child's serum, plasma, red blood cell (RBC) and mononuclear cell (MNC) fatty acid (FA) composition and the development of atopic diseases and asthma during childhood from earlier studies organized by different type of study designs: Cohort study, randomized controlled trial (RCT) and case-control study.

Author, country	Study design (Child's age at the end of follow-up)	Subjects	Intervention	Outcome	Results
Furuhjelm <i>et al.</i> 2011, Sweden	RCT (24 mo)	145 pregnant women at risk of having an allergic infant	Fish oil group (1.6 g EPA and 1.1g DHA) or placebo group, from 25 <sup>th</sup> week of gestation to 3–4 mo breastfeeding	Clinical examination, severity of eczema by SCORAD, blood samples for IgE from infants (delivery, 3, 12 and 24 mo), plasma n-3 polyunsaturated fatty acids (PUFA)	High levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in maternal and infant plasma phospholipids inversely associated with IgE-positive disease and severity of the allergic phenotype
Almqvist <i>et al.</i> 2007, Sweden	RCT (18 mo, 3 and 5 yrs)	616 mother-child pairs at risk for allergy, randomized at 36 weeks gestation to	n-3 group: oil capsule 500 mg tuna fish oil, high n-3, low n-6 intake	Wheeze and eczema by questionnaire and atopy by skin prick test (SPT) (18 mo, 3 and 5 yrs)	Plasma n-3 PUFA levels not associated with atopic diseases at 5 yrs of age, overall FA exposure not

Mirshahi <i>et al.</i> 2004, Australia	RCT (18 mo)	616 women with a family history of atopy	control group: 500 mg Sunola oil, low n-3, high n-6 intake N-3 PUFA (daily tuna fish oil supplement, margarine, cooking oils) or control group (placebo, margarine, cooking oils)	Asthma and atopy at 18 mo	Higher plasma n-3 PUFA inversely associated with asthma	associated with respiratory or allergic outcomes	any
Dunder <i>et al.</i> 2001, Finland	Cohort (6 yrs)	770 children aged 3, 6, 9, 12, 15 and 18 yrs	-	Childhood diseases during follow-up	EPA, DHA and myristic acid lower in serum cholesteryl esters in children with atopic disease		
Yen <i>et al.</i> 2008 Japan	Case-control	35 children with atopic dermatitis (AD), 35 age-matched children with allergic rhinitis, asthma or both, 31 non-atopic controls	-	Atopic asthma	Atopic children had higher levels of linoleic acid (LA) and lower levels of its metabolites		

Early life diet and asthma, with an emphasis on the role of fatty acids

Hwang <i>et al.</i> 2007, South Korea	Case- control	108 children aged 4–6 yrs	AD, allergic rhinitis or asthma	Total RBC N-3 PUFA lower in children with AD, n-6 PUFA, ratio of n-6 to n-3, higher saturated fatty acid (SAFA) and arachidonic acid (AA) and lower total and n-3 PUFA in atopic children
Bolte <i>et al.</i> 2006, Germany	Nested case- control and cross- sectional	185 cases, 341 controls; 432 in cross-sectional study, aged 8–11	Wheeze and asthma by questionnaire, lung function measurements, SPT	Serum cholesteryl ester LA positively associated with current asthma and decreased forced expiratory volume (FEV1), AA positively associated with current asthma and inversely with FEV1
Laitinen <i>et al.</i> 2006, Finland	Case- control	6 atopic eczema, 6 non-atopic eczema, 19 controls	Atopic or non-atopic eczema	LA, n-6 PUFA and total PUFA higher in cheek cells in infants with atopic eczema, lower serum GLA in atopic and non-atopic eczema, EPA higher in atopic eczema compared with controls
Focke <i>et al.</i>	Case-	22 children with AD, -	Atopic dermatitis	Plasma GLA levels lower in

2005, Austria	control	14 controls (1–16 yrs)	(criteria of Hanifin and Raika, SPT, IgE levels)	eczema patients with elevated IgE and in children with allergic rhinitis and asthma, LA levels lower in atopic controls
Kankaanpää <i>et al.</i> 2001, Finland	Case-control (3 mo)	20 allergic and 20 healthy mothers and their infants 10 atopic and 10 non-atopic/group of mothers	Atopy	Serum GLA lower in breast milk of allergic mothers and in phospholipids of allergic infants, n-6 PUFA elevated in other serum lipid fractions in atopic infants
Yu <i>et al.</i> 1996, Sweden	Case-control	22 cases, 23 controls, aged 12–15	Asthma and/or allergic dermatitis	Levels of DHA and total n-3 PUFA in serum phospholipids lower and the ratio of n-6 to n-3 PUFA higher in allergic children, levels of docosapentaenoic acid (DPA) and AA:dihomo-γ-linolenic acid (DHGLA) lower in SPT-positive children
Leichsenring <i>et al.</i> 1995, Germany	Case-control	17 patients with asthma, 10 controls	Atopic asthma	Higher plasma phospholipid levels of LA and lower AA levels in atopic

Griese <i>et al.</i> 1990, Germany	Case-control	11 allergic asthmatic cases and 10 non-allergic controls aged 4–13 yrs; 14 allergic and non-allergic children with acute asthma attack who received glucocorticoids	-	Allergic asthma, acute asthma attack	children EPA elevated in plasma and MNC in allergic asthma, levels of EPA correlated with higher serum total IgE, no significant changes in FA after glucocorticoids
<hr/>					
AA=arachidonic acid, AD=atopic dermatitis, DHA=docosahexaenoic acid, DHGLA=dihomo- $\gamma$ -linolenic acid, DPA=docosapentaenoic acid, EPA=eicosapentaenoic acid, FA=fatty acids, FEV=forced expiratory volume, FFQ=food frequency questionnaire, GLA= $\gamma$ -linolenic acid, IgE=immunoglobulin E, LA=linoleic acid, MNC=mononuclear cell, PUFA=polyunsaturated fatty acids, RBC=red blood cell, SAFA=saturated fatty acids, SPT=skin prick test					



## 2.4 Diet in early life

The composition of the mothers diet during pregnancy is important both to the mother and to the growing fetus *in utero*. A large body of evidence indicates that maternal nutrition during pregnancy has a critical impact on fetal growth, the child's neural and cognitive development, and later risk of chronic diseases (Koletzko *et al.* 2008). It is proposed that early life diet, beginning already during pregnancy, has long-lasting effects on the child's later health (Barker *et al.* 1999). A suboptimal growth environment *in utero* may affect the organogenesis and the development and function of hormonal balance, and also have an effect on the risk of cardiovascular disease, type 1 and 2 diabetes mellitus and allergic diseases (Barker *et al.* 1999, Duijts *et al.* 2012). The quality of the diet during pregnancy is important to meet the energy and nutrient requirements for the growth and development of the fetus and the well-being of the mother (Koletzko *et al.* 2008). During pregnancy, the absorption of the nutrients is more effective, so the amount of extra energy needed is quite small. The placenta regulates both the nutritional intake of the fetus and the metabolism of the mother (Koletzko *et al.* 2008).

### 2.4.1 Diet during pregnancy, lactation and infancy during the study period in Finland: Recommendations and actual diet.

In Finland, all pregnant women and preschool children have regular visits to maternity and well-baby clinics. One of the most important roles of these clinics is to provide current and updated information on nutritional guidance for families. Finnish dietary recommendations are based on the regularly updated Nordic dietary recommendations (Nordic Council of Ministers 2014). The DIPP Nutrition Study was launched in the 1990s and the Nordic Dietary recommendations have been updated three times after this launch. The latest recommendations were published in 2014.

In 1989, pregnant and lactating women were advised to use low-fat or fat-free milk and milk products. Foods containing PUFA (vegetable oils, margarines and fish) were recommended (Hasunen *et al.* 1989). In 1997, these recommendations persisted; consumption of fish 2–3 times weekly was recommended (Hasunen *et al.* 1997). In 2004, fat-spreads containing >60% fat were recommended (Hasunen *et al.* 2004). The latest dietary recommendations published in 2014 focus on the quality of the diet. The suggested intake of folate and vitamin D among pregnant women is higher compared with earlier recommendations (Nordic Council of Ministers 2014).

In 1989, 6–12 months of total breastfeeding was endorsed. The recommended age to start complementary feeding was 3–6 months and the introduction of sour milk

products was recommended at ten months. Milk containing 1.5% fat was recommended for children aged one year or older. Only a small amount of visible fat on bread was recommended (Hasunen *et al.* 1989). In 1997, fat-free or low-fat milk was endorsed for children over one year of age. For children consuming fat-free milk, 2–3 teaspoons of vegetable oil per day was recommended. (Hasunen *et al.* 1997). Vitamin D 10 ug per day was recommended until the age of three years. Exclusive breastfeeding for the first six months and a total period of twelve months of breastfeeding were endorsed (Hasunen *et al.* 2004). As of 2014, daily vitamin D supplementation is recommended throughout life.

The actual diet of pregnant Finnish women has not met the recommendations. Only one fourth of the pregnant mothers eat the recommended amounts of vegetables, fruits and berries (Arkkola *et al.* 2006). The intakes of folate, vitamin D and iron were below recommendations (Erkkola *et al.* 1998, Piirainen *et al.* 2006, Prasad *et al.* 2010). Only half of pregnant women eat fish at least once a week (Erkkola *et al.* 1998, Arkkola *et al.* 2006). On the other hand, the intake of meat and meat products is six times higher than recommended (Prasad *et al.* 2010). The consumption of SAFA is exceeded, while the consumption of PUFA falls below the recommendations. Dietary counselling has improved diet during pregnancy in the direction of the recommendations (Kinnunen *et al.* 2014).

Adequate nutrition is the basis for growth, development and the well-being of the child. Breast-milk is the best nutrition for the newborn. Breast milk includes all the nutrients needed during the first months of life except vitamin D. The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life and the introduction of complementary foods after that. The duration of total breastfeeding should be 12 months (WHO 2003). Recently, the American Academy of Pediatrics (AAP) and the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) published new guidelines concerning early nutrition and allergy prevention in infants and children. These organizations also recommended exclusive breastfeeding for the first six months (Agostoni *et al.* 2008, Greer *et al.* 2008). Complementary feeding should be started at the age of 17–26 weeks (Agostoni *et al.* 2008, Greer *et al.* 2008, Fleischer *et al.* 2013). It has been proposed that starting complementary foods at the age of 4–6 months under the protective effect of breastfeeding has beneficial effects on the prevention of food allergies (Greer *et al.* 2008, Fleischer *et al.* 2013, Nwaru *et al.* 2013).

The actual diet of Finnish children aged 0–6 years was studied in 2010 (Kyttälä *et al.* 2010). The mean duration of exclusive breastfeeding was 1.4 months, and that of total breastfeeding seven months (Erkkola *et al.* 2010). The consumption of

vegetables, fruits and berries fell below the recommendations while the consumption of cereals, meat and milk products exceeded the recommendations (Kyttälä *et al.* 2010). The use of margarines and fish was low. The quality of the diet became poorer after the children began to eat same food with the family. The intake of sugar, chocolate and sweets increased at the age of two years. Children aged 2–6 years consumed more sucrose, salt and SAFA, and less PUFA, vitamin D, vitamin E and iron (Kyttälä *et al.* 2010). The children in day care had healthier eating habits compared with children cared for at home (Lehtisalo *et al.* 2010).

#### **2.4.2 The role of early life diet in the development of asthma and atopic diseases**

##### **Diet during pregnancy and lactation**

Intrauterine environment and antenatal exposures have an impact on the development of allergic diseases. Maternal diet during pregnancy can regulate the early immunological reactions of the fetus and infant, thus influencing the balance between inflammatory and anti-inflammatory reaction types (Devereux *et al.* 2002). The mother's prenatal diet might influence the development of childhood asthma by affecting fetal airway development and by modulating interactions between allergens and the immune system (Devereux *et al.* 2006, Olsen *et al.* 2008). In addition, it is possible that prenatal n-3 PUFA exposure alters the eicosanoid metabolism of the neonate (Blumer *et al.* 2007).

Table 3 summarizes the earlier studies on maternal food, especially fat and FA, intake during pregnancy and lactation on the development of allergic diseases (food allergy, hay fever and asthma) and atopic sensitization during childhood. Epidemiological studies suggest the protective association of maternal fish intake during pregnancy with atopic outcomes in children (Salam *et al.* 2005, Calvani *et al.* 2006, Sausenthaler *et al.* 2007, Willers *et al.* 2007, Romieu *et al.* 2009, Saadeh *et al.* 2013). Maternal intake of fish during lactation has not been associated with a risk of atopic diseases (Hoppu *et al.* 2005). On the other hand, Furuhejm *et al.* reported a protective effect from maternal long-chain n-3 PUFA supplementation in pregnancy and lactation on IgE-positive eczema and food allergy in infants up to one year of age (Furuhejm *et al.* 2009). Studies have also reported a reduced sensitization to common food allergens and a reduced prevalence and symptoms of atopic dermatitis during the first year of life with a possible persistence to adolescence with a reduction on the prevalence of asthma, hay fever and atopic eczema (Kremmyda *et al.* 2011). Maternal fish oil supplementation during pregnancy resulted in higher n-3 PUFA and lower n-6 PUFA status in neonates (Dunstan *et al.* 2003) and higher

levels of docosahexaenoic acid (DHA) both in maternal and cord blood (Krauss-Etschmann *et al.* 2007). Early fish oil provision during pregnancy, lactation and infancy is associated with immunological changes in cord blood and such changes may persist until adolescence (Kremmyda *et al.* 2011).

The fetus receives n-6 and n-3 PUFA via the placenta while breast milk is an important source of these FA after birth (Kankaanpää *et al.* 2001, Cunningham *et al.* 2009). The placental transport processes favor n-3 PUFA over n-6 PUFA (Cunningham *et al.* 2009). The FA composition of breast milk is adjusted by both maternal diet and maternal FA stores.

Only a few studies have investigated the associations between maternal dietary FA composition during lactation and the risk of asthma in the offspring. By contrast, the relationships between the duration of breastfeeding, maternal allergen avoidance during lactation the FA composition of breast milk and atopic diseases in childhood have been investigated extensively (Friedman *et al.* 2005, Kneepkens *et al.* 2010, Silvers *et al.* 2012). According to those studies, a smoke-free environment, exclusive breastfeeding for 4–6 months and the postponement of supplementary feeding until four months of age considered effective in allergy prevention. There is no evidence on maternal dietary restrictions during pregnancy or lactation on prevention of allergies in the offspring (Kneepkens *et al.* 2010).

The process of the literature review is shown in Appendix 1.

**Table 3.**

The role of maternal dietary fat and fatty acid intake during pregnancy and lactation and the risk of asthma and atopic diseases during childhood. Summary of evidence from earlier studies organized by different type of study designs: randomized controlled trial (RCT), cohort study and case-control study.

Reference, country	Study design (Child's age at the end of the follow-up)	Subjects	Dietary intervention/exposure	Outcome	Results
Palmer <i>et al.</i> 2012, Australia	Double-blind RCT (1 yr)	706 pregnant women with a family history of allergy	21 weeks gestation to delivery, n-3 group: 800 mg docosahexaenoic acid (DHA) and 100 mg eicosapentaenoic acid (EPA) from fish oil daily, control group: vegetable oil (mix of rapeseed, sunflower and palm oil)	Immunoglobulin (IgE)-associated allergic disease: atopic eczema or food allergy	E No reduction in IgE-related allergic diseases
Noakes <i>et al.</i> 2012, UK	RCT (6 mo)	123 pregnant women with a family history of allergy	20 weeks gestation to delivery, fish group: 2 x 150 g salmon per week (3.45 g EPA plus DHA), control group: normal diet	Cord blood fatty acids (FA), mononuclear cell cytokine and IgE production, leukocyte phenotypes, serum total IgE at birth and 6 mo, clinical outcomes at 6 mo	Cytokine production lower in fish group but no effect on allergic diseases

Furuhjelm <i>et al.</i> 2011, Sweden	Double-blind RCT (24 mo)	145 pregnant women with a family history of allergy	25 weeks gestation through lactation (2–4 mo), n-3 group: 1.6 g EPA and 1.1 g DHA from fish oil daily, control group: soy oil	Serum IgE for specific allergens at 24 mo, cumulative incidence (0–24 mo) of positive skin prick tests (SPT), allergic symptoms, IgE-positive atopic dermatitis and other IgE-positive allergic diseases	No preventive effect on clinical allergic symptoms, the decrease in cumulative incidence of IgE-associated atopic disease remained until 2 yrs of age. Higher proportions of EPA and DHA in maternal and infant serum phospholipids associated with less IgE-positive disease and less severe allergic phenotype
Manley <i>et al.</i> 2011, Australia	Double-blind RCT (18 mo)	657 preterm infants whose mothers were supplemented with fish oil or placebo	From birth until expected date of delivery, high DHA group: breast milk or preterm formula with 0.85-1% of FA as DHA, control group: breast milk or standard formula with 0.25–0.35% of DHA	Incidence of bronchopulmonary dysplasia (BPD), Structured parental interview at 12 and 18 mo about hay fever, atopic dermatitis, asthma, food allergy, hospital readmissions	High DHA reduced incidence of hay fever in boys

Linnamaa <i>et al.</i> 2010, Finland	RCT (2 yrs)	313 mother-child pairs	Blackcurrant seed oil (BCSO) or olive oil as a placebo from 8–16 <sup>th</sup> week of pregnancy to mothers and until the child is 2 yrs old	AD by serum total IgE	BCSO transiently reduced the prevalence of atopic dermatitis at 12 mo but not at 24 mo
Furuhjelm <i>et al.</i> 2009, Sweden	Double-blind RCT (3, 6 and 12 mo)	145 pregnant women with a family history of allergy	25 weeks gestation through lactation (2–4 mo), n-3 group: 1.6 g EPA and 1.1 g DHA from fish oil daily, control group: soy oil	Serum IgE for specific allergens at 3 and 12 mo, IgE-positive atopic dermatitis, food allergy, SPT 6 and 12 mo	Prevalence of food allergy and IgE-associated atopic eczema lower in n-3 group
Olsen <i>et al.</i> 2008, Denmark	RCT (16 yrs)	533 women	30 wks gestation until delivery, 1 g capsule containing fish oil (2.7 g n-3 PUFA), olive oil or no oil	Asthma diagnosis from registry data	N-3 polyunsaturated FA (PUFA) in late pregnancy inversely associated with the risk of asthma
Lauritzen <i>et al.</i> 2005, Denmark	RCT (2 yrs)	122 lactating mothers	4.5 g/day fish oil (1.5 g n-3 PUFA) or olive oil first 4 mo of lactation	Cytokine production	Increased interferon-gamma production in 2.5-yr-old children in fish oil group (faster maturation of the immune system)

Dunstan <i>et al.</i> 2004, Australia	Double-blind RCT (20 wks gestation to 6 wks)	83 women pregnant	20 wks delivery, daily 4 g fish oil capsules or placebo (4 g olive oil capsules)	Erythrocyte phospholipids FA in maternal blood (20, 30, 37 weeks gestation and 6 weeks after delivery) and cord blood	Maternal EPA and DHA significantly higher, n-6 PUFA lower, same findings from cord blood in fish oil group
Dunstan <i>et al.</i> 2004, Australia	Double-blind RCT (20 wks gestation to 3 days)	83 women pregnant	20 wks delivery, daily 4 g fish oil capsules or placebo (olive oil)	Breast milk collected 3 d postpartum, FA, IgA, CD14 and cytokines	DHA and EPA levels higher, AA lower, IgA positively correlated with DHA but inversely with LA, CD14 positively correlated with EPA
Dunstan <i>et al.</i> 2003, Australia	Double-blind RCT (20 wks gestation to delivery)	83 women pregnant	20 wks delivery, 3.7g n-3 PUFA or placebo daily	Cord blood cytokine production and total IgE-levels	n-3 PUFA supplementation increased n-3 PUFA levels in neonatal erythrocyte membranes and decreased neonatal plasma and cell membrane IL-13 levels; no difference in levels of IgE



Dunstan <i>et al.</i> 2003, Australia	Double-blind RCT (1 yr)	98 pregnant women	20 wks gestation to delivery, fish oil (3.7 g n-3 PUFA per d) or placebo	Neonatal PUFA levels and immunologic response to allergens at birth	In fish oil group higher proportions of n-3 PUFA in neonatal erythrocyte membranes, cytokine responses lower, 3 times less likely to have a positive SPT for egg at the age of 1 yr, less severe atopic eczema
Miyake <i>et al.</i> 2014, Japan	Prospective cohort (2 yrs)	1354 mother-child pairs	Diet history questionnaire, dietary habits during the preceding months before delivery	Questionnaire on allergic disorders	Higher maternal intake of total dairy products, cheese, yogurt and calcium during pregnancy inversely associated with infantile eczema, doctor-diagnosed asthma and atopic eczema
Bunyavanich <i>et al.</i> 2014, USA	Prospective cohort (mean 7.9 yrs)	1277 mother-child pairs	FFQ during 1 <sup>st</sup> and 2 <sup>nd</sup> trimester, maternal intake of common childhood food allergens	Questionnaire on food allergy, asthma, allergic rhinitis, atopic dermatitis, serum IgE	Higher intake of peanut, milk and wheat during pregnancy inversely associated with mid-childhood allergy and asthma

Early life diet and asthma, with an emphasis on the role of fatty acids

Maslova <i>et al.</i> 2013, Denmark	Prospective cohort (18 mo, 7 yrs)	28 936 mothers	Fish intake >2–3 times per wk at 12 and 30 weeks gestation	Self-report and registry data on hospitalizations and prescribed medications: ever wheeze, recurrent wheeze, ever asthma, rhinitis at 18 mo and 7 yrs	High fish intake inversely associated with both early and ever asthma in 7-yr old children
Maslova <i>et al.</i> 2012, Denmark	Prospective cohort (18 mo, 7 yrs)	61 pregnant women	909 Milk and yoghurt consumption during mid-pregnancy (25 <sup>th</sup> week gestation)	Childhood asthma and allergic rhinitis	Maternal low-fat yoghurt intake positively associated with childhood asthma and rhinitis, whole milk protective for early outcomes only
Pelé <i>et al.</i> 2013, France	Prospective cohort (2 yrs)	3421 pregnant women	Fish and shellfish consumption by food frequency questionnaire (FFQ)	Wheeze, eczema, food allergy by questionnaire	Moderate fish intake associated with a lower risk of wheeze, shellfish consumption increased the risk of food allergy
Leermakers <i>et al.</i> 2013,	Prospective cohort (4 yrs)	2976 mother-child pairs	Fish consumption during first trimester of pregnancy	Wheezing and eczema by questionnaire	Shellfish consumption positively associated

The Netherlands					with wheezing and eczema, fatty fish consumption with eczema only
Miyake <i>et al.</i> 2013, Japan	Prospective cohort (23–29 mo)	1354 mother-child pairs	Maternal fat intake during pregnancy	Wheeze and eczema by questionnaire	High maternal intake of EPA and DHA reduced the risk of infantile wheeze
Soto-Ramírez <i>et al.</i> 2012, USA	Prospective cohort (12 mo)	231 pregnant women	24 colostrum and 78 breast milk samples, n-3 and n-6 PUFA content	Asthma-like symptoms (AS) at 6 and 12 mo and SPT 12 mo	High levels of total n-6 PUFA in breast milk associated with an increased risk of AS; high levels of n-3 PUFA decreased the risk of atopy
Nwaru <i>et al.</i> 2012, Finland	Prospective cohort (5 yrs)	2441 mother-child pairs	A validated FFQ, 8 <sup>th</sup> mo of pregnancy	Allergic rhinitis and atopic eczema	Maternal consumption of butter and a higher ratio of n-6 to n-3 positively associated and PUFA and $\alpha$ -linolenic acid) ALA inversely associated with rhinitis

Nwaru <i>et al.</i> 2011, Finland	Prospective cohort (5 yrs)	652 child pairs	mother- pairs	A validated FFQ, 3 <sup>rd</sup> month of lactation	Allergic sensitization	Butter and saturated fatty acids (SAFA) intake positively, while margarine inversely associated with a sensitization to wheat allergen, margarine intake inversely associated with sensitization to birch allergen
Sausenthaler <i>et al.</i> 2011, Germany	2 prospective birth cohorts GINIplus and LISApplus (2 and 6 yrs)	2252 infant pairs	mother- pairs	FFQ during last 4 wks of pregnancy	Allergic sensitization against food and inhalant allergens, doctor-diagnosed and symptomatic eczema	High intake of margarines and vegetable oils positively associated with risk of allergies, especially eczema, high intake of fish inversely associated with eczema, high intake of deep-fried vegetable fat positively related to sensitization to inhalant allergens
Thjis <i>et al.</i>	Prospective	315	mother- pairs	Breast milk FA at 1 mo	Questionnaire on atopic	n-3 and ruminant FA

2011, The Netherlands	follow-up (24 mo)	infant pairs	postpartum	outcomes, serum total IgE and IgE for specific allergens at 1 and 2 yrs of age	associated with a decreased risk of atopic diseases
Castro-Rodriguez <i>et al.</i> 2010, Chile	Prospective cohort (12 mo)	1409 infants (mean age 16.6±2.5 mo) and their mothers	FFQ during pregnancy	Wheezing during 1 <sup>st</sup> year of life	Adherence to Mediterranean diet and using olive oil associated with reduced wheezing
Nwaru <i>et al.</i> 2010, Finland	Prospective cohort (5 yrs)	931 mother-child pairs	FFQ 8 <sup>th</sup> pregnancy	Allergic sensitization (Allergen-specific IgE)	PUFA did not reduce the risk for allergic sensitization
Øien <i>et al.</i> 2009, Norway	Prospective cohort (2 yrs)	3086 children	Fish and fish oil intake during pregnancy and first year of life	Parental asthma and eczema at 2 yrs of age	Fish consumption during infancy negatively associated with eczema
Miyake <i>et al.</i> 2009, Japan	Prospective cohort (2 yrs)	763 mother-child pairs	Diet history questionnaire (DHQ), anytime during pregnancy	Wheeze and eczema from ISAAC questionnaire	ALA and DHA inversely associated with wheeze, n-6 PUFA and LA positively associated with eczema
Sausenthaler	Prospective	2641 mother-child pairs	FFQ during last 4 weeks of pregnancy	Sensitization for food	High intake of n-6

<i>et al.</i> 2009, Germany	birth cohort (2 yrs)	child pairs	pregnancy	and inhalant allergens, eczema	PUFA increase, and high intake of n-3 PUFA decrease the risk of allergic sensitization and eczema
Chatzi <i>et al.</i> 2008, Greece	Prospective cohort (6.5 yrs)	460 mother-child pairs	FFQ during pregnancy	Wheeze and atopy	Adherence to Mediterranean diet during pregnancy inversely associated with asthma-like symptoms and atopy
Romieu <i>et al.</i> 2007, Mexico	Prospective cohort (6 yrs)	462 pregnant women	FFQ during pregnancy	Atopic diseases by positive SPT	Fish intake during pregnancy inversely associated with atopic diseases
Willers <i>et al.</i> 2007, Scotland	Prospective cohort (5 yrs)	1212 mother-child pairs	FFQ, reflecting intake 2–3 months prior to 32 weeks gestation	Parental report on asthma, atopic eczema, wheezing, hay fever, spirometry and SPT on small number of children	Maternal intake of oily fish $\geq 1/\text{wk}$ inversely associated with eczema
Calvani <i>et al.</i>	Retrospective	295 offspring	Standardized	Atopy (positive SPT for	In non-allergic mothers,

2006, Italy	cohort	of allergic mothers and 693 offspring of non-allergic mothers	questionnaire, maternal use of fish, margarine and butter	eight inhalant allergens and two foods)	fish intake inversely associated with SPT positivity, similar trend for inhalants, in the whole population similar trend
Hoppu <i>et al.</i> 2005, Finland	Cohort (1 yr)	34 atopic mothers and their infants	Breast milk samples 1 mo postpartum; Maternal dietary intake (4-day food records) and questionnaire on maternal dietary habits (including fish consumption frequency) 1 mo postpartum	Clinical examination 1, 3, 6, 12 mo, SPT 12 mo, Atopic dermatitis during the 1 <sup>st</sup> year of life (Hanifin criteria)	Maternal fish consumption during pregnancy not related to breast milk EPA content; the ratio of SAFA/PUFA higher in breast milk consumed by infants developing atopic dermatitis; Total n-3 PUFA and EPA lower in breast milk of mothers whose infants developed atopic dermatitis
Dotterud <i>et al.</i> 2013, Norway	Case-control (2 yrs)	1374 cases, 833 controls	FFQ during pregnancy	Asthma, atopic eczema by questionnaire	Increased intake of oily fish during pregnancy inversely associated with asthma

Kitz <i>et al.</i> 2006, Germany	Case-control (12 mo)	131 infants (exclusively breastfed 58, combined-fed 53, never breastfed 20)	$\gamma$ -linolenic acid (GLA) supplement or placebo to mothers and newborns up to 6 mo	Atopic dermatitis (AD), S-IgE	24/131 developed AD; GLA-supplementation could not prevent AD, AD lowest in infants never breastfed
Salam <i>et al.</i> 2005, USA	Case-control (5 yrs)	279 cases, 412 controls	Telephone interview on fish consumption	Doctor-diagnosed asthma	Oily fish consumption during pregnancy inversely associated with the risk of asthma in the offspring, eating fish sticks positively associated with the risk of asthma

AD=atopic dermatitis, ALA= $\alpha$ -linolenic acid, AS=asthma-like symptoms, BPD=brochopulmonary dysplasia, BSCO=blackcurrant seed oil, DHA=docosahexaenoic acid, DHQ=diet history questionnaire, EPA=eicosapentaenoic acid, FA=fatty acids, FFQ=food frequency questionnaire, GLA= $\gamma$ -linolenic acid, IgE=Immunoglobulin E, PUFA=polyunsaturated fatty acids, SAFA=saturated fatty acids, SPT=skin prick test.



## Diet during childhood

Earlier studies have reported associations between a higher consumption of fruits, vegetables, nuts, wholegrain cereals and full-fat dairy products during childhood and a decreased risk of asthma (Willers *et al.* 2011, Barros *et al.* 2011, Peroni *et al.* 2012). The Mediterranean diet has been protective against childhood wheeze and asthma in many studies (Chatzi *et al.* 2007, Arvaniti *et al.* 2010, Nagel *et al.* 2010, Antó 2012, Garcia-Marcos *et al.* 2013).

Table 4 summarizes earlier studies on food, especially fat and FA, intake during infancy and childhood and the risk of allergic diseases (food allergy, hay fever and asthma) and atopic sensitization. Some studies on fish intake during infancy and childhood observed beneficial associations (Dunder *et al.* 2001, Nafstad *et al.* 2003, Andreasen *et al.* 2005, Kim *et al.* 2005, Hodge *et al.* 2006, Kull *et al.* 2006, Chatzi *et al.* 2007), some observed harmful associations (Hijazi *et al.* 2000, Takemura *et al.* 2002), and some observed no associations (Farchi *et al.* 2003, Wijga *et al.* 2003, Alm *et al.* 2009). Based on the epidemiological data available, the findings are inconsistent. In a systematic review of double-blind, placebo-controlled randomized trials, no clear evidence was achieved for an association between supplementation of the child with n-3 and n-6 PUFA at any age and the prevention of asthma, allergic rhinitis, atopic eczema or food allergy (Anandan *et al.* 2009).

Maternal fish oil supplementation during pregnancy results in immunological changes in cord blood and the infant's higher n-3 PUFA status. In addition, fish oil supplementation during infancy and childhood increases the status of these FA in the blood, but the results on allergic diseases are inconsistent and the clinical significance and persistence of these changes is not known (Kremmyda *et al.* 2011). The inconsistent results may be due to differences in sample sizes, selection of study populations, definitions of atopic diseases and study designs. There is no evidence of the efficacy of n-3 PUFA supplementation in the treatment of already existing asthma (Hodge *et al.* 1998, Nagakura *et al.* 2000, Kremmyda *et al.* 2011).

A cross-sectional study from ISAAC phase II with 50,004 children from twenty countries showed that the consumption of fruits, fish and cooked green vegetables is associated with a lower risk for current wheeze and lifetime prevalence of asthma, while hamburger consumption was associated with an increased risk of asthma (Nagel *et al.* 2010). In a prospective birth cohort study from the Netherlands, fruit consumption in early life and also longitudinal fruit consumption were both associated with a decreased prevalence of asthma and sensitization to inhalant allergens by eight years of age (Willers *et al.* 2011). A prospective birth cohort study

from Sweden reported that regular fish consumption during infancy decreased the risk of allergic diseases up to the age of twelve years (Magnusson *et al.* 2013).

**Table 4.**

Intake of foods and dietary fat and fatty acids, during childhood and the development of asthma and atopic diseases in earlier studies organized by different types of study designs: randomized controlled trial (RCT), prospective cohort study, case-control study and cross-sectional study.

Author, country	Study design (Child's age at the end of follow-up)	Subjects	Dietary intervention/exposure	Outcome	Results
D'Vaz <i>et al.</i> 2012, Australia	RCT (12 mo)	420 allergic mothers	N-3 group: 280 mg docosahexaenoic acid (DHA) and 110 mg eicosapentaenoic acid (EPA) per day from fish oil, Control group: olive oil until 6 mo	Plasma and erythrocyte fatty acids (FA), immune responses to allergens at 6 mo	Increased n-3 polyunsaturated FA (PUFA) levels, lowered allergen-specific Th2-responses, elevated Th1-polyclonal responses
D'Vaz <i>et al.</i> 2012, Australia	RCT (12 mo)	420 allergic mothers	N-3 group: 280 mg DHA and 110 mg EPA per day from fish oil, control group: olive oil until 6 mo	Allergic diseases at 6 and 12 mo, SPT at 12 mo	DHA and EPA levels higher and erythrocyte arachidonic acid (AA) levels lower in fish oil group, n-3 PUFA not associated with allergic outcomes
Manley <i>et al.</i>	Double-blind	657 preterm	From birth until expected	Incidence	DHA reduced hay fever

2011, Australia	RCT (18 mo)	infants whose mothers were supplemented with fish oil or placebo	date of delivery, high DHA group: breast milk or preterm formula with 0.85–1% of FA as DHA, control group: breast milk or standard formula with 0.25–0.35% of DHA	bronchopulmonary dysplasia (BPD), structured parental interview at 12 and 18 mo about hay fever, atopic dermatitis, asthma, food allergy, hospital readmissions	in boys
Peat <i>et al.</i> 2004, Australia	RCT (3 yrs)	616 children antenatally	Active intervention group: 500 mg tuna fish oil from 6 mo of age, canola-based oils and spreads control group: Canola oil capsules and soybean-based polyunsaturated oils and margarines	Questionnaire on allergic symptoms and sensitization	Reduction in cough in atopic children but not in non-atopic children in active group, no significant difference in wheeze
Van Gool <i>et al.</i> 2003, The Netherlands	Double-blind RCT (1 yr)	118 infants at high familial risk	Borage oil supplement (100 mg $\gamma$ -linolenic acid, GLA) or placebo daily for first 6 mo	Atopic dermatitis (AD) by SCORAD and total serum IgE at 1 yr of age	GLA does not prevent AD as reflected by serum IgE, but it tends to alleviate the severity of AD in later infancy
Hodge <i>et al.</i> 1998,	Double-blind RCT	39 asthmatic children aged	Fish oil capsules plus canola oil and margarine	Plasma FA, stimulated TNF- $\alpha$ production,	N-3 supplementation increased plasma n-3

Australia	(3 and 6 mo)	8–12 yrs	(n-3 group) or safflower oil capsules plus sunflower oil and margarine (n-6 group) 3-6 mo	circulating eosinophil numbers and lung function	levels, reduced TNF- $\alpha$ but had no effect on severity of asthma
Magnusson <i>et al.</i> 2013, Sweden	Prospective birth cohort (12 yrs)	3285 children	Fish consumption	Allergic diseases	Regular consumption during infancy inversely associated with the risk of allergic diseases up to 12 yrs
Alm <i>et al.</i> 2009, Sweden	Prospective cohort (6 and 12 mo)	8176 infants	Age at introduction of solid foods	Food allergy and AD, questionnaire	Introduction of fish before 9 mo inversely associated with the risk of AD
Goksor <i>et al.</i> 2011, Sweden	Prospective cohort (4.5 yrs)	8176 infants	Age at introduction of solid foods	Wheezing, questionnaire	Introduction of fish before 9 mo inversely associated with the risk of recurrent wheeze
Alm <i>et al.</i> 2011, Sweden	Prospective cohort (4.5 yrs)	8176 infants	Age at introduction of solid foods	Allergic questionnaire	Introduction of fish before 9 mo inversely associated with the risk

Hesselmar <i>et al.</i> 2010, Sweden	Prospective cohort (18 mo)	184 infants with a history of allergy)	Feeding practices at 6 and 12 mo by parent interview	Parent interviews of allergy symptoms at 6 and 12 mo, clinical and laboratory examination for allergy at 18 mo	Early fish introduction inversely associated with the risk of eczema and asthma, no impact on sensitization	of allergic rhinitis
Øien <i>et al.</i> 2010, Norway	Prospective cohort (6 yrs)	3086 infants	Diet and other exposure in pregnancy through 1 yr by questionnaire	Questionnaire at 2 yrs on allergic diseases	Fish consumption in infancy inversely associated with the risk of eczema	
Virtanen <i>et al.</i> 2010, Finland	Prospective cohort (5 yrs)	1302 infants	Feeding practices at 3, 6, 12 and 24 mo by questionnaire	ISAAC-based questionnaire on history and symptoms of asthma, allergic rhinitis and atopic eczema at 5 yrs of age	Early introduction of oats inversely associated with persistent asthma and early introduction of fish inversely associated with allergic rhinitis	
Kull <i>et al.</i> 2006, Sweden	Prospective cohort (4 yrs)	4089 infants	Feeding practices at ages 2 mo and 1, 2 and 4 yrs by questionnaire	Clinical investigation and IgE to common food and inhalant allergens	Regular fish consumption before age 1 yr reduced the risk of allergic diseases and sensitization to food and	

Sausenthaler <i>et al.</i> 2006, Germany	Prospective cohort (2 yrs)	2582 children	Fat spread intake by FFQ and open questions	Eczema, sensitization	allergic	inhalant allergens
Wijga <i>et al.</i> 2003, The Netherlands	Prospective cohort (3 yrs)	2978 children	Food consumption at 2 yrs of age by FFQ	Questionnaire on allergic symptoms at 3 yrs of age		Predominant margarine consumption over butter consumption increased risk for allergic sensitization and eczema
Nafstad <i>et al.</i> 2003, Norway	Prospective cohort (4 yrs)	2531 children	Introduction of fish in the diet during 1 <sup>st</sup> year of life	Asthma and rhinitis	allergic	In preschool children, frequent consumption of cow's milk products inversely associated with the risk of asthma
Farchi <i>et al.</i> 2003, Italy	Prospective cohort (1 yr)	4104 children aged 6–7 yrs	Dietary intake including fish, pasta with fish by FFQ	ISAAC questionnaire on wheezing and asthma		Fish consumption during the first yr of life inversely associated with the risk of asthma and rhinitis
Birch <i>et al.</i>	Retrospective,	89 exclusively	DHA/AA formula or	Diagnosis of	atopic	No associations between fish intake and asthma Reduced risk for

2010, US	cohorts from 2 double-blind RCTs (3 yrs)	formula-fed infants	control formula with no long-chain PUFA fed from 1 <sup>st</sup> week of life until 12 mo	symptoms respiratory from medical records	and infections	asthma, AD/wheezing/allergy and upper respiratory infections in DHA/AA group
Kunitsugu <i>et al.</i> 2012, Japan	Case-control	135 students with eczema, 136 with asthma and 137 healthy controls	Dietary intake questionnaire	by Total erythrocyte FA concentrations	IgE, membrane	Fish consumption associated with lower prevalence of eczema but not asthma; erythrocyte EPA levels inversely associated with eczema
Oddy <i>et al.</i> 2004, Australia	Case-control (8 yrs)	2602 children	Dietary intake questionnaire	by Current asthma at 6 or 8 yrs of age		Higher n-6 and n-3 reduced the risk for asthma
Nakamura <i>et al.</i> 2013, Japan	Cross-sectional	452 children aged 3-6 yrs	Children's diet by 3-day food records	Questionnaire on children's demographics		Higher intakes of vitamins C and E inversely associated with asthma, no associations between PUFA or fruits and asthma



Loss <i>et al.</i> 2011, The Netherlands	Cross-sectional	8334 children aged 6–12 yrs living in rural areas of Germany, Austria and Switzerland	Farm milk consumption and other exposures	consumption farm-related	IgE from blood samples, ISAAC-based questionnaire on allergy symptoms	Raw milk consumption inversely associated with asthma and hay fever; increased levels of whey proteins bovine serum albumin (BSA), $\alpha$ -lactalbumin and $\beta$ -lactoglobulin inversely associated with asthma
Miyake <i>et al.</i> 2011, Japan	Cross-sectional	23388 children aged 6–15 yrs	Diet history during the preceding month by validated diet history questionnaire (BDHQ)	the month by diet history questionnaire (BDHQ)	ISAAC-based questionnaire on symptoms of eczema and/or rhinoconjunctivitis	Consumption of n-3 and n-6 PUFA, especially linoleic acid (LA) and ALA positively associated with eczema, AA inversely related to eczema and rhinoconjunctivitis
Nakamura <i>et al.</i> 2013, Japan	Cross-sectional	452 children aged 3–6 yrs	Dietary intake by food record	by 3-day	Questionnaire on asthma diagnosis	No associations of FA with asthma
Rodríguez-Rodríguez <i>et al.</i> 2010,	Cross-sectional	638 Spanish school children aged 8–13 yrs	Dietary intake by food record	by 3-day	Questionnaire on personal and health information	Increased intake of saturated FA (SAFA), myristic and palmitoleic

Early life diet and asthma, with an emphasis on the role of fatty acids

Spain					acids and butter positively associated with the risk of current asthma
Nagel <i>et al.</i> 2010, Germany	Cross-sectional	50 004 children from ISAAC phase two, aged 8–12 yrs	Dietary questionnaire	intake by Parental and SPT	Fruit, fish and cooked green vegetable intake and Mediterranean diet associated with a lower prevalence of current wheeze and lifetime prevalence of asthma
Waser <i>et al.</i> 2006, Switzerland	Cross-sectional	14893 children aged 5–13 yrs	Dietary questionnaire	intake by Allergen-specific IgE	Farm milk consumption protective against asthma and allergy
Andreasyan <i>et al.</i> 2005, Australia	Cross-sectional	499 children aged 8 yrs	Parental report of fish intake	SPT, asthma, hay-fever, wheeze, eczema by ISAAC questionnaire	Fish intake associated with a decreased risk of asthma and hay-fever linked to ryegrass-pure sensitization
Takemura <i>et al.</i> 2002, Japan	Cross-sectional	1673 asthmatic students, 22109 controls (aged	Questionnaire on food consumption and questions on frequency of	Current asthma	Higher fish intake positively associated with current asthma

	6–15)	foods eaten	
Bolte <i>et al.</i> 2001, Germany	Cross-sectional study 2348 children aged 5–14 yrs	Information on type of fat used during preceding 12 mo	Allergic sensitization by specific serum IgE  Margarine consumption associated with allergic sensitization in boys, no relation with hay fever or asthma
Hijazi <i>et al.</i> 2000, Saudi Arabia	Cross-sectional 1444 children (mean age 12 (SD 1)); 114 asthmatics, 202 controls	Questionnaire on food intake	ISAAC questionnaire on asthma and wheezing, atopy by SPT  Negative association between fish intake and asthma; fast food increased the risk for wheezy illness, intake of milk and vegetables inversely associated with asthma
Hodge <i>et al.</i> 1996, Australia	Cross-sectional study 574 children	Questionnaire on oily fish consumption	Wheeze, airway hyperresponsiveness (AHR), atopy; current asthma=recent wheeze+AHR  Oily fish consumption protective against asthma in childhood

AA=arachidonic acid, AD=atopic dermatitis, AHR=airway hyperresponsiveness, ALA= $\alpha$ -linolenic acid, BDHQ=validated diet history questionnaire, BPD=bronchopulmonary dysplasia, BSA=bovine serum albumin, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, FA=fatty acids, FFQ=food frequency questionnaire, GLA= $\gamma$ -linolenic acid, IgE=immunoglobulin E, LA=linoleic acid, PUFA=polyunsaturated fatty acids, SAFA=saturated fatty acids, TNF=tumor necrosis factor.

## 2.5 Summary of the literature review

The increased prevalence of asthma and atopic diseases in Western countries during the past decades parallels an increase in the dietary intake of n-6 PUFA, and a decrease in the intake of n-3 PUFA and SAFA. N-6 PUFA are precursors for proinflammatory eicosanoids and may play an important role in the development of IgE-mediated allergic diseases, such as asthma, through AA and its metabolites. N-3 PUFA have anti-inflammatory properties and may antagonize the effects of AA and thus prevent the development of asthma.

Earlier studies suggest that foods rich in antioxidants and n-3 PUFA protect against childhood asthma. A healthy maternal diet rich in antioxidants and n-3 PUFA during pregnancy may protect against asthma and atopic diseases in the offspring. Many epidemiological studies have reported protective associations between maternal fish or fish oil intake during pregnancy and allergic outcomes in the offspring.

Exclusive breastfeeding for four to six months and postponement of solid foods until four months of age are considered effective in preventing allergic diseases in children.

Earlier studies have also reported associations between higher consumption of fruits, vegetables, nuts, whole-grain cereals and whole-fat dairy products during childhood and a decreased risk of asthma. In addition, the Mediterranean diet has been protective against childhood wheeze and asthma. The evidence on the associations between fish intake and asthma risk is inconsistent.

Serum FA composition is modulated by diet, metabolic processes, and the atopic diseases process itself. In early infancy, higher serum levels of n-3 PUFA have been associated with a decreased risk of atopy, but the evidence is inconsistent. There is also evidence of lower serum proportions of LA metabolites, dihomog- $\gamma$ -linolenic acid,  $\gamma$ -linolenic, and AA, in children with atopic disease. This may represent the impaired ability to convert LA to its longer-chain metabolites, or increased use of these FA in atopic children.

The results of the earlier studies are inconsistent. In addition, the role of the diagnosed CMA and dietary restrictions due to it has not been taken into account, and this subject requires further study.

### 3 Aims of the study

The purpose of the study was to assess the associations of early life nutrition, especially fats and FA, with the development of childhood asthma. Our specific aims were to study the associations:

- Between maternal consumption of FA during pregnancy and the development of asthma in the offspring by the age of five years (Study I);
- Between maternal intake of fats and FA during lactation and the development of asthma in the offspring by the age of five years (Study II);
- Between longitudinal food consumption during infancy and childhood and the development of asthma by the age of five years (Study III);
- Between longitudinal serum FA proportions during childhood and the development of asthma by the age of five years (Study IV).

# 4 Materials and methods

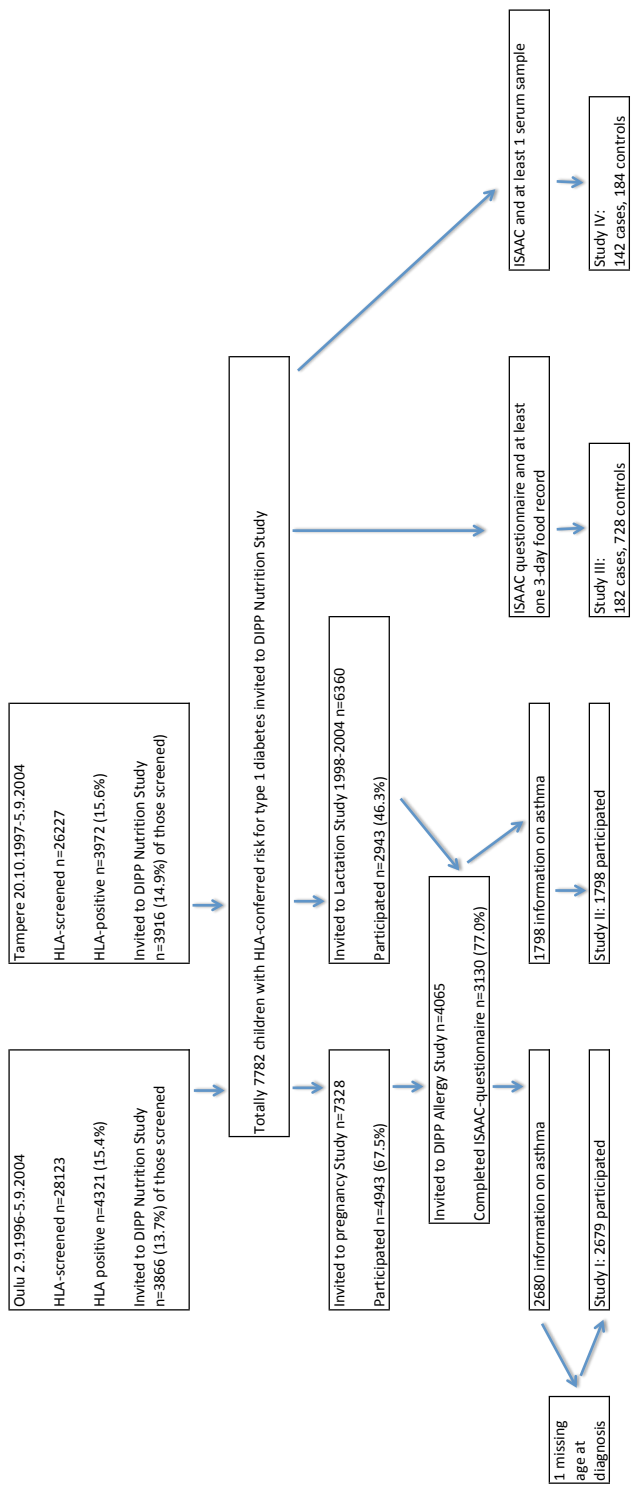
## 4.1 Subjects and study design

In the Type 1 Diabetes Prediction and Prevention (DIPP) Project, a population-based cohort study, infants at three university hospitals in Finland (Turku, Tampere and Oulu), were screened for human leukocyte antigen (HLA)-DQB1-conferred genetic susceptibility to type 1 diabetes with the use of cord blood samples after parental informed consent (Kupila *et al.* 2001). Infants carrying a high or moderate genetic risk (HLA-DQB1\*02/0302 heterozygous and DQB1\*0302/x-positive subjects; x stands for homozygosity or neutral alleles) for type 1 diabetes were invited to participate in the study (15% of those screened). The aim of the study was to monitor diabetes-associated antibodies, growth, and environmental exposures (Kupila *et al.* 2001). Infants with severe congenital malformations or diseases, parents of non-Caucasian origin and parents who did not have a working knowledge of Finnish, Swedish or English were excluded from the study.

The DIPP Nutrition Study was started within the framework of the larger DIPP in September 1996 in Oulu and in October 1997 in Tampere (Virtanen *et al.* 2006). The focus of the Nutrition Study was to investigate the impact of maternal nutrition during pregnancy and lactation, and the child's diet, on the development of advanced beta cell autoimmunity, type 1 diabetes, asthma, allergic diseases and obesity in the offspring. Altogether 54 350 children (28 123 in Oulu and 26 227 in Tampere) were screened for HLA-conferred risk for type 1 diabetes. Of those screened, 8293 children (15.2%) were HLA-positive (4321 in Oulu and 3972 in Tampere). A total of 7782 children (93.8% of those HLA-positive) formed the DIPP Nutrition Study cohort (3866 in Oulu and 3916 in Tampere).

The children still taking part in the dietary follow-up at the age of five years (n=4065) were invited to the DIPP Allergy Study to complete a questionnaire on the child's history of allergic symptoms and asthma. Of those, 3737 (92%) participated. 3130 (84% of those who participated) completed the ISAAC-questionnaire. 2908 of these children (77% of those who participated) had the information on asthma available.

Figure 4 shows the flow of subjects in studies I-IV.



**Figure 4.** The flow of subjects in studies I-IV.

### **Study I: Dietary fatty acids during pregnancy and the risk of asthma in the offspring**

The pregnancy study started among mothers of children born late in 1997 in Oulu and Tampere (7238 children invited). Maternal dietary data during pregnancy (181-item FFQ covering the eighth month of pregnancy) was requested for children born between December 13 1997 and October 31 2004 at Oulu University Hospital and October 20 1997 and September 5 2004 at Tampere University Hospital. Mothers of 4943 children participated in the pregnancy study (68% of those invited). Allergy data (ISAAC-based written questionnaire at the age of five years) were available for 2908 of those children. Of those children for whom maternal dietary data was available, 2680 also had information on their history of asthma. One case child was excluded because of missing data on the age of asthma diagnosis, which would have been required for statistical analyses, resulting in 2679 subjects available in the analysis.

### **Study II: Dietary fat and fatty acid intake during lactation and the risk of asthma in the offspring**

The lactation study started among mothers of children born in 1998 (6360 children invited). Only mothers still breastfeeding the child at three months were invited to the lactation study. Mothers of 2943 children participated in this study (46% of those invited). We analyzed children born between 1998 and 2004 in Tampere and Oulu. Of children with data on maternal lactation diet (181-item FFQ covering the third month of lactation) available (n=2943), 1798 also had information on asthma. The children were partly same as in pregnancy study.

### **Study III: Early life diet and asthma: The role of cow's milk allergy**

This study was nested within the cohort of children born between 1996 and 2004 at Tampere (n=3916) and Oulu University Hospitals (n=3866). To be eligible for the study, all subjects had to have completed the ISAAC-based written questionnaire and have at least one three-day food record available for analysis. For each case, there were four randomly selected controls free of asthma until the age when the case was diagnosed with asthma. The controls matched for gender, genetic risk group for type 1 diabetes, research area (Tampere or Oulu) and time of birth ( $\pm 3$  months), were randomly selected. The control must have been followed up for asthma until the age of five years. The present analysis comprised 182 cases and 728 matched controls. Seventy-six children served as controls for more than one case but



were included only once in the total number of controls. Fifteen of the controls became a case at a later date and were counted only as cases. A total of 104 children (11.4%) had the diagnosis of CMA at the age of six months, 96 (10.5%) at the age of twelve months and 63 (6.9%) at 24 months of age.

#### **Study IV: Cow's milk allergy and the association between serum fatty acids and childhood asthma risk**

This study was nested within the cohort of children born between 1996 and 2004 in Tampere (n=3916) and Oulu (n=3866). The selection of subjects was made with same principles as in the previous study. All subjects must have at least one serum sample available. Two matched controls were selected for each case. The control must have been followed up until the age of five years and could not come from the same family as the case. The controls were independently selected so that the same child could be a control for more than one case child and a child who was already a control could become a case at a later time point. Altogether, 142 asthma cases and 284 matched controls formed the final sample for the present study. Eleven children served as controls for more than one case but were included only once in the total number of control subjects. Four of the control subjects became cases at a later date and were counted only as cases. Fifty-nine of the children (13.8%) in this study had the diagnosis of CMA.

## **4.2 Outcome assessment**

### **4.2.1 Asthma diagnosis (Study I-IV)**

When the child was five years, the parents completed a slightly modified, ISAAC-based (Asher *et al.* 1998) written questionnaire, where the child's history of allergic diseases was investigated. The asthma component of the questionnaire was validated against the anti-asthmatic medication reimbursement data of the Finnish Social Insurance Institution (Nwaru *et al.* 2011). Two combined questions (any wheezing symptom or use of asthma medication during the preceding 12 months *plus* doctor-diagnosed asthma) were validated against a valid reimbursement with a purchase of at least one anti-asthmatic medication during a 12-month period (Nwaru *et al.* 2011). The Finnish ISAAC questionnaire was highly valid and therefore, in this study, asthma was based on a written questionnaire as follows: a doctor-diagnosed asthma *plus* either any wheezing symptom or the use of asthma medication during the preceding 12 months before the completion of the questionnaire. Age of the child at asthma diagnosis was determined by the question: "at what age was asthma diagnosed by a doctor?" Based on the questionnaire, all the children still had asthma at the age of five years indicating persistent asthma.

#### 4.2.2 Immunoglobulin E (IgE) measurements

The specific IgE concentrations were measured from serum samples obtained at the age of five years by using ImmunoCAP fluoroenzyme immunoassay (Phadia Diagnostics, Uppsala, Sweden). IgE concentrations were measured for the following allergens: egg, cow's milk, fish, wheat, house dust mite, cat, Timothy-grass and birch. The IgE -positivity cutoff-value was set at  $\geq 0.35$  kU/l. Atopy was defined as a sensitization ( $\geq 0.35$  kU/l) to any of these allergens (Nwaru *et al.* 2011).

#### 4.2.3 Food allergy diagnosis

Food allergies and special diets were inquired from the parents at study center visits with open questions at the ages of 3 and 6 months and 1 and 2 years, and also with a structured questionnaire at the ages of 3, 4, 5 and 6 years. Parents responded to open questions on CMA in their children at ages of six months, and one and two years, and to a structured questionnaire at three years of age. An open question was: "Does the child follow a special diet?" At three years, the question regarding CMA were: "Has the child ever had cow's milk allergy?". "On whose initiative was the diet started (parents or healthcare professionals)?", and "What was the child's age at the beginning and the end of the diet?" (Tuokkola *et al.* 2010). The structured questionnaire was validated against hospital records (Tuokkola *et al.* 2010) and against information on CMA diagnosis from the registers of the Finnish Social Insurance Institution (Tuokkola *et al.* 2008). The diagnoses of food allergies come both from the structured questionnaire and from the registers of the Finnish Social Insurance Institution (Tuokkola *et al.* 2008). In addition to CMA, the occurrence of lactose intolerance, coeliac disease, any food allergies, vegetarian diet and elimination of any allergenic foods were asked in a structured questionnaire. The starting and ending ages of special diets were enquired separately.

### 4.3 Dietary methods

#### 4.3.1 Food frequency questionnaire

Dietary intakes during pregnancy were determined using a validated, self-administered, semi-quantitative 181-item Food frequency questionnaire (FFQ) (Erkkola *et al.* 2001). The FFQ was designed to assess the total maternal diet over a period of one month. The frequency of food consumption (number of times per day, per week or per month) was assessed as common serving sizes (portion sizes based on natural units and commonly used portions identified in earlier Finnish dietary studies) (Prasad *et al.* 2010). The mothers were asked to circle the corresponding picture of the amount of fat spread they used on one slice of bread. Frequency of consumption was also asked. The use of margarines was classified depending on their fat content: margarines containing 70–80% or 30–60% fat. Brand names were also given. The questionnaire was designed to reflect specifically the food

consumption of pregnant Finnish women. The pregnancy FFQ assessed the diet of the mother during the 8<sup>th</sup> -month of pregnancy, which is the month just prior to the maternity leave in Finland. Mothers were asked to supply information on the use of nutrient supplements over the whole duration of the pregnancy. The use of nutrient supplements during the whole pregnancy and lactation was asked from the mothers in a separate question, along with information on brand names, manufacturers and the amount of use per day, week or month (Prasad *et al.* 2010).

The pregnancy FFQ was returned to the study center and checked by a trained study nurse or doctor at the child's three-month visit. All returned FFQ forms were further processed by a trained nutritionist. FFQs with missing data for ten or more food items were rejected. Food consumption data was analyzed using a continuously updated software program (Fineli) developed at the National Institute for Health and Welfare, Helsinki, and converted into a daily food diary from which the mean daily use of foods and nutrients was calculated. Information on fats used in cooking and baking and oils used for salad dressing was asked and included in the nutrient calculations (Prasad *et al.* 2010).

Dietary intake during lactation was assessed using a validated, self-administered, semi-quantitative 181-item FFQ during the third month of lactation. The FFQ was designed to assess the maternal total diet (intake of foods and nutrients) over a period of one month (Erkkola *et al.* 2001). The diet during the third month of lactation was queried by an FFQ given to the mothers at the child's three-month visit to the study center and returned at the child's six-month visit. The processing of the FFQ was the same as in the pregnancy FFQ.

#### 4.3.2 Three-day food record

Information on the child's diet was inquired by means of three-day food records at the ages of 3, 6 and 12 months and thereafter annually up to six years of age (Virtanen *et al.* 2012). The three-day food records, which were completed by the parents at home, were checked at the child's visits to the study clinic by the trained research staff. The information on food records was entered to the database by a trained nutritionist using a booklet of food portion sizes, household measures, food product brochures and an annually updated national food database, Fineli ([www.fineli.fi](http://www.fineli.fi)), to estimate the contents and amounts used (Virtanen *et al.* 2012). The absolute daily consumption of different foods and food groups were calculated. Special diets were also asked. The food record data were analyzed until the time when the case was diagnosed with asthma.

#### 4.3.3 Age-specific dietary questionnaires

Data on the timing of infant feeding were collected up to two years of age (Nwaru *et al.* 2013). The diet of each child was assessed by means of age-specific dietary questionnaires at the ages of 3, 6, and 12 months, with a follow-up form on the age at introduction of new foods to the diet. Each of the age-specific questionnaires inquired about the pattern of breastfeeding, the use of infant formula and cow's milk, the use of vitamin preparations, and the food items the child had received to date. The form on the age at introduction of new foods was kept and filled in by the families up to two years of age (Nwaru *et al.* 2010).

#### 4.3.4 Estimation of breast milk intake

Among breastfed children, the quantity of breast milk consumed was estimated using calculations based on growth and expected energy requirements (EER) (Schoen *et al.* 2009).

**Table 5.** Expected energy requirement (EER) equals to total energy requirement (TEE) plus energy needed for deposition (Schoen *et al.* 2009). EER for infants and young children,

Age (months)	TEE (kcal)	Energy deposition (kcal)
0–3	$89 \times \text{weight of infant (kg)} - 100$	175
4–6	$89 \times \text{weight of infant (kg)} - 100$	56
7–12	$89 \times \text{weight of infant (kg)} - 100$	22

Estimated energy from breast milk is equal to EER minus energy from food records. Based on this, the average amount of breast milk consumed per day was calculated (Schoen *et al.* 2009).

### 4.4 Serum fatty acids

Non-fasting serum samples for FA analyses were drawn by venipuncture annually at the ages of one to five years. The samples were centrifuged and the serum was protected from light and stored at  $-70^{\circ}\text{C}$  until analyzed (Uusitalo *et al.* 2008, Uusitalo *et al.* 2011). The samples from each case-control sets were analyzed up to the time when the case child was diagnosed with asthma. The relative content of individual FA is presented as percentage of total FA in serum.

Fat was extracted from 50 µl of serum with dichloromethane-methanol (2:1, v:v). Total fatty acids were methylated with acidic methanol (5% weight H<sub>2</sub>SO<sub>4</sub>). The percentage composition of methylated total FA from 14:0 to 22:6n-3 were determined by a HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with Chemstation (version A.06.03) with a DB225 column (30-m long I.D. 0.32 mm, phase layer 0.25 µm; Agilent J&W GC, Palo Alto, CA, USA), using split injection and hydrogen as the carrier gas. A temperature program for 160°C to 230°C was used. The percentage composition of FA methyl esters was normalized to 100%. Between-series variability of control samples was 2-7% for FA peaks over 1% and 6-18% for smaller peaks. Altogether 17 individual FA levels were measured (Virtanen *et al.* 2010, Uusitalo *et al.* 2011).

#### 4.5 Sociodemographic and other background characteristics of the study population

Information on the child's gender, maternal age and maternal and paternal vocational education was obtained from a structured written questionnaire completed by the parents after delivery. Information on birth weight, mode of delivery (vaginal or caesarean section), duration of gestation, number of previous deliveries and maternal smoking during pregnancy as well as diagnoses of any infections of the mother during pregnancy and delivery, or infections and antibiotic treatment of the child during the first week of life, was received from the Medical Birth Registries of Oulu and Tampere University Hospitals. Information on maternal or paternal asthma or allergic rhinitis and dogs at home during the first year of life were determined from the ISAAC questionnaire at the age of five years. Information on duration of total breastfeeding and the ages of introduction of special solid foods were obtained from the written questionnaire. The information on the diagnosis of CMA was obtained both from the written questionnaire and from the registers of the Finnish Social Insurance Institution.

#### 4.6 Statistical methods

##### 4.6.1 Logistic regression and cox proportional hazards regression (Study I and II)

Logistic regression was used to assess the associations between background characteristics and the risk of asthma. Cox proportional hazards regression was applied to assess the association between maternal intake of FA and dietary fats during pregnancy and lactation and the risk of asthma in the children at the age of event time. The dietary intake of FA was energy-adjusted by the Willett's residual method (Willett 1998). When assessing associations with the food variables, energy

was also included in the model. All dietary variables were divided into quartiles. The lowest and highest quartiles were compared with the combined mid half as the reference category. The selection of confounding variables was based on biological plausibility, and on their relations with the outcome in the present and earlier studies. The associations between confounding variables and outcome have been studied using conditional logistic regression analysis. In the adjusted models, the following covariates were selected: maternal age, mode of delivery, gestational age, number of siblings, birth weight, sex of the child, delivery hospital area, maternal smoking during pregnancy, parental asthma or allergic rhinitis, maternal vocational education, pets (pets inside the house or domestic animals in a building outside the house) at home, farming, contact with cowsheds (or other building where animals were kept) during the first year of life and the duration of total breastfeeding. The statistical significance of the dietary variables was tested with the Wald Chi-Square test.

When taking the sibling effect into account in the Cox regression, the associations between consumption of fats and FA and the risk of asthma remained the same. Maternal sources of dietary FA were calculated in grams or milligrams per day and in percentage of FA intake. Analyses were computed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA, Version 9.1.). The clustering effect of siblings was taken into account in the Cox regression by clustering by family size in STATA (College Station, Texas, USA, Version 9.2.).

Mean food and nutrient intakes during pregnancy and lactation were calculated in relation to energy intake to compare the intakes between pregnancy and lactation. Paired samples t-test was used to compare the intakes. The nutrient variables were calculated as a percentage of energy to better describe the quality of the diet, not depending on the amounts consumed.

#### **4.6.2 Generalized estimating equation framework, conditional logistic regression (Study III and IV)**

The estimation of risk was based on a nested case-control design set up within the cohort. The standard procedure for estimation in nested case-control designs is to use conditional logistic regression analysis (Borgan *et al.* 1995, Borgan 2003). However, in our setting, exposure variables were measured repeatedly at different ages before the onset of the disease. Measurements of the same individual tend to be correlated, and this was accounted for by using a generalized estimating equation (GEE) framework with the sandwich estimator of variance to estimate regression coefficients (Craiu *et al.* 2008). This approach results in consistent estimates of regression coefficients, valid standard errors (SE), and narrower confidence intervals (CI) than does an approach that uses only one measurement per individual.

The differences between cases and controls in the background characteristics were tested with the Wald test obtained from the conditional logistic regression analysis. The associations between serum FA variables and asthma were analyzed longitudinally until the diagnosis of asthma. The analyses were also stratified by atopy, which was defined as IgE positivity and IgE negativity. Interactions between serum FA variables and age at the time of the measurement, as well as interactions between duration of breastfeeding and serum FA variables, were tested using interaction tests with a significance level of 0.05. Serum FA variables were used both as continuous and categorical variables (aggregated into quartiles, the intermediate half used as a reference category) in the analysis. Because no indication for non-linearity of the associations was observed, only results for the continuous serum FA variables are presented.

Median serum FA proportions were calculated separately in children with and without CMA and the Mann-Whitney U test was used to examine the differences. CMA was used as one of the putative confounders to study the associations of CMA-related changes in serum FA profiles on the results.

When studying inference methods, the conditional logistic regression model with longitudinal data was used to analyze the associations between longitudinal food intakes and the risk of asthma. The analyses were also stratified by IgE positivity (i.e. sensitization ( $\geq 0.35$  kU/l) to any of the tested allergens). Interactions between food intake variables and CMA and cow's milk use were assessed using interaction tests with a significance level of 0.05, and stratification. Food intake variables were used as continuous in the analysis and they were  $\log(x+1)$  transformed to obtain normality. The differences between cases and controls in the background characteristics were assessed using conditional logistic regression analysis. When the proportion of users was  $\geq 25\%$ , median food intakes were calculated separately in children with and without CMA, and the Mann-Whitney U test was used to examine the differences. When the proportion of food users was  $\leq 25\%$ , food intakes were categorized into users and non-users, and the Chi-Square or Fisher's exact test were used to examine the differences.

The following covariates were used as putative confounders: gestational age, maternal age, maternal smoking during pregnancy, number of siblings, maternal asthma or allergic rhinitis, paternal asthma or allergic rhinitis, birth weight, mode of delivery, duration of total breastfeeding, maternal vocational education, dogs at home during the first year of life and CMA. In study III, we also used the age at introduction of cereals, egg and fish in tertiles as putative confounders. The selection of confounding variables was based on their relations with the endpoint in the present study and also based on findings from earlier studies. It has earlier been shown that among children who have wheezing before the age of three years, a

family history of asthma in a sibling or parent, a history of food allergy or an itchy rash, and exposure to tobacco smoke were all independently associated with current asthma at school age (Csonka *et al.* 2000).

#### 4.7 Ethical issues

In the DIPP Study, newborn infants were screened for genetic susceptibility to type 1 diabetes and this may limit the generalizability of the results to general population. However, the benefits of the screening for individual study subjects were expected: the study subjects were given more accurate medical and nutritional guidance and follow-up. This study was observational cohort study, not an intervention study. The only invasive measures were blood tests for IgE and FA analyses, and these are routine and safe procedures. The study was approved by local ethical committees of the hospitals and a written informed consent was obtained from the parents.



# 5 Results

## 5.1 Characteristics of the study population

Among children with endpoint data (n=2908), 175 (6%) had asthma. Median (IQR) age at asthma diagnosis was 2.5 (1.1) years. Median duration of exclusive breastfeeding was 2.3 (1.8) months and that of total breastfeeding 9.2 (4.7) months. Median age of mothers at the time of delivery was 30.1 (4.9) years and median gestational age of the children 39.8 (1.5) weeks. Mean (SD) number of previous deliveries was 0.9 (1.2).

Of the background characteristics studied, reported CMA, maternal and paternal asthma or allergic rhinitis, duration of total breastfeeding less than 9 months, as well as antibiotic treatment of the child during the first week of life were associated with an increased risk of asthma in the offspring. Full-term pregnancy and female gender were associated with a reduced risk of asthma. Introduction of oats, wheat, barley or rye cereals at the age of 5–5.5 months, that of fish at 6.1–8 months and that of egg at 10 months or earlier were associated with a decreased risk of asthma.

## 5.2 Maternal fat and fatty acid intake during pregnancy and lactation and the risk of asthma in the offspring by the age of five years (Study I and II)

Low maternal intakes of ALA and total n-3 PUFA during pregnancy were associated with an increased risk of asthma in the offspring, while AA was associated with a decreased risk of asthma in the offspring. Higher intakes of SAFA and palmitic acid were associated with a decreased risk of asthma in the offspring. Associations between the main dietary sources of these fatty acids and childhood asthma could not be demonstrated (Tables 6 and 7). The main sources of n-6 PUFA and LA among pregnant women in our study were oils, margarines, and red meat and meat products. The main sources of n-3 PUFA were oils, margarines and fish, and the main sources of SAFA were milk and milk products.

During lactation, higher maternal use of margarines was associated with a marginally increased risk of asthma in the offspring. There was no evidence of an association of intake of n-3 PUFA with the risk of asthma (Tables 6 and 7).

We compared the differences in maternal food consumption between pregnancy and lactation. The consumption of oils, margarines, butter and butter-spreads and meat

and meat products in relation to energy intake was higher during lactation, while those of milk and milk products were higher during pregnancy. The energy intake from carbohydrate as a percentage of energy intake was higher during pregnancy. The reverse was true for protein, total fat, SAFA, MUFA, total PUFA, n-6 PUFA, EPA and DHA.

**Table 6.** The risk of asthma (hazard ratios (HR) and 95% confidence interval (CI)) associated with maternal fatty acid intake during pregnancy and lactation.

FA in quarters (ranges in grams)	Pregnancy Adjusted <sup>1</sup> HR (95% CI)	p-value
SAFA		<b>0.008</b>
Lowest quartile (<32.93)	1.28 (0.86–1.90)	
2 Mid quartiles (32.93–53.84)	1	
Highest quartile (>53.84)	<b>0.55 (0.34–0.89)</b>	
PUFA		0.173
Lowest quartile (<10.71)	1.32 (0.89–1.97)	
2 Mid quartiles (10.71–17.01)	1	
Highest quartile (>17.01)	0.84 (0.53–1.32)	
Palmitic acid		
16:0		<b>0.003</b>
Lowest quartile (<15.53)	1.30 (0.87–1.94)	
2 Mid quartiles (15.53–24.82)	1	
Highest quartile (>24.82)	<b>0.50 (0.30–0.82)</b>	
N-6 PUFA		<b>0.039</b>
Lowest quartile (<8.03)	1.41 (0.95–2.08)	
2 Mid quartiles (8.03–12.75)	1	
Highest quartile (>12.75)	0.75 (0.47–1.20)	
LA		
18:2n-6		0.067
Lowest quartile (<7.78)	1.30 (0.87–1.92)	
2 Mid quartiles (7.78–12.36)	1	
Highest quartile (>12.36)	0.71 (0.45–1.13)	
ARA		
20:4n-6		<b>0.025</b>
Lowest quartile (<0.06)	<b>0.52 (0.32–0.84)</b>	
2 Mid quartiles (0.06–0.11)	1	
Highest quartile (>0.11)	0.77 (0.51–1.17)	
N-3 PUFA		<b>0.036</b>
Lowest quartile (<2.24)	<b>1.66 (1.11–2.48)</b>	
2 Mid quartiles (2.24–3.84)	1	
Highest quartile (>3.84)	1.09 (0.70–1.70)	
ALA		
18:3n-3		<b>0.022</b>
Lowest quartile (<1.83)	<b>1.70 (1.14–2.53)</b>	
2 Mid quartiles (1.83–3.18)	1	
Highest quartile (>3.18)	1.06 (0.68–1.65)	

EPA		
20:5n-3		0.604
Lowest quartile (<0.04)	1.09 (0.72–1.65)	
2 Mid quartiles (0.04–1.13)	1	
Highest quartile (>1.13)	0.84 (0.54–1.31)	
DHA		
22:6n-3		0.467
Lowest quartile (<0.10)	0.80 (0.52–1.23)	
2 Mid quartiles (0.10–0.32)	1	
Highest quartile (>0.32)	0.83 (0.53–1.29)	

FA in quarters (ranges in grams)	Lactation Adjusted <sup>2</sup> HR (95% CI)	p-value <sup>3</sup>
SAFA		0.451
Lowest quartile (<28.39)	0.91 (0.50–1.68)	
2 Mid quartiles (28.39–46.58)	1	
Highest quartile (>46.58)	1.42 (0.78–2.59)	
PUFA		0.289
Lowest quartile (<9.37)	0.61 (0.31–1.20)	
2 Mid quartiles (9.37–15.13)	1	
Highest quartile (<15.13)	1.10 (0.63–1.91)	
Palmitic acid		
16:0		0.818
Lowest quartile (<15.11)	1.04 (0.56–1.94)	
2 Mid quartiles (15.11–19.45)	1	
Highest quartile (>19.45)	1.21 (0.68–2.15)	
N-6 PUFA		0.345
Lowest quartile (<7.09)	0.62 (0.32–1.19)	
2 Mid quartiles (7.09–11.31)	1	
Highest quartile (>11.31)	1.00 (0.57–1.76)	
LA		
18:2n-6		0.256
Lowest quartile (<6.83)	0.58 (0.30–1.14)	
2 Mid quartiles (6.83–10.99)	1	
Highest quartile (>10.99)	1.03 (0.59–1.79)	
ARA		
20:4n-6		0.378
Lowest quartile (<0.06)	0.65 (0.35–1.23)	
2 Mid quartiles (0.06–0.10)	1	
Highest quartile (>0.10)	1.03 (0.60–1.78)	

N-3 PUFA	0.252
Lowest quartile (<1.94)	0.61 (0.32–1.18)
2 Mid quartiles (1.94–3.37)	1
Highest quartile (>3.37)	0.74 (0.41–1.35)
ALA	
18:3n-3	0.146
Lowest quartile (<1.56)	0.59 (0.32–1.11)
2 Mid quartiles (1.56–2.80)	1
Highest quartile (>2.80)	0.66 (0.37–1.20)
EPA	
20:5n-3	0.732
Lowest quartile (<0.04)	0.79 (0.43–1.47)
2 Mid quartiles (0.04–0.12)	1
Highest quartile (>0.12)	1.04 (0.57–1.93)
DHA	
22:6n-3	0.643
Lowest quartile (<0.09)	0.81 (0.44–1.48)
2 Mid quartiles (0.09–0.31)	1
Highest quartile (>0.31)	1.15 (0.63–2.09)

<sup>1</sup>Adjusted for sex, area of birth, duration of gestation, maternal age, maternal vocational education, maternal smoking during pregnancy, number of siblings, parental asthma, parental allergic rhinitis, birth weight, mode of delivery, pets at home, farming, contact with cowsheds during the first year of life and the duration of total breastfeeding.

<sup>2</sup>Adjusted for sex, area of birth, duration of gestation, maternal age, maternal vocational education, maternal smoking during pregnancy, number of siblings, maternal allergy, paternal allergy, birth weight, mode of delivery, duration of breastfeeding, pets at home, farming, contact with cowsheds during the first year of life and fat consumption during pregnancy.

<sup>3</sup>p for overall test

ALA= $\alpha$ -linolenic acid, ARA=arachidonic acid, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, FA=fatty acid, LA=linoleic acid, PUFA=polyunsaturated fatty acid, SAFA=saturated fatty acid.

**Table 7.** The risk of asthma in the offspring (hazard ratios (HR) and 95% confidence interval (CI)) associated with maternal consumption of certain fat-containing foods during pregnancy and lactation.

Food variable (ranges in grams)	Pregnancy Adjusted <sup>2</sup> HR (95% CI)	p-value
Oils		0.576
Lowest quartile (<7.3)	1.07 (0.69–1.65)	
2 Mid quartiles (7.3–16.6)	1	
Highest quartile (>16.6)	1.26 (0.82–1.94)	
Margarines		0.274
User (>15.00)	0.82 (0.58–1.17)	
Non-user (0)	1	
Butter and butter-oil spreads		0.126
Lowest quartile (<4.6)	1.41 (0.91–2.19)	
2 Midquartiles (4.6–19.8)	1	
Highest quartile (>19.8)	1.47 (0.96–2.23)	
Industrial fat <sup>4</sup>		0.309
Lowest quartile (<11.4)	1.40 (0.91–2.16)	
2 Mid quartiles (11.4–29.7)	1	
Highest quartile (>29.7)	1.09 (0.70–1.70)	
Fish		0.861
Lowest quartile (<10.7)	1.12 (0.74–1.70)	
2 Mid quartiles (10.7–32.0)	1	
Highest quartile (>32.0)	1.06 (0.68–1.65)	
Red meat and meat products		0.469
Lowest quartile (<88.5)	0.76 (0.47–1.22)	
2 Mid quartiles (88.5–156)	1	
Highest quartile (>156)	1.06 (0.67–1.67)	
Milk and milk products		0.282
Lowest quartile (<547)	1.17 (0.78–1.77)	
2 Mid quartiles (547–1100)	1	
Highest quartile (>1100)	0.73 (0.44–1.21)	

Food variable (ranges in grams)	Lactation Adjusted <sup>3</sup> HR (95% CI)	p-value <sup>5</sup>
Oils		0.942
Lowest quartile (<6.2)	1.05 (0.56–1.98)	
2 Mid quartiles (6.2–15.7)	1	
Highest quartile (>15.7)	0.92 (0.51–1.67)	

Margarines		<b>0.047</b>
User (>0)	<b>1.96 (1.01–3.82)</b>	
Non-user (0)	1	
Butter and butter-oil spreads		0.667
Lowest quartile (<3.8)	0.99 (0.53–1.83)	
2 Midquartiles (3.8–19.3)	1	
Highest quartile (>19.3)	1.33 (0.70–2.53)	
Industrial fat <sup>4</sup>		0.753
Lowest quartile (<9.3)	0.83 (0.45–1.55)	
2 Mid quartiles (9.3–26.4)	1	
Highest quartile (>26.4)	1.13 (0.61–2.11)	
Fish		0.962
Lowest quartile (<8.8)	1.01 (0.56–1.82)	
2 Mid quartiles (8.8–29.2)	1	
Highest quartile (>29.2)	1.09 (0.58–2.06)	
Red meat and meat products		0.118
Lowest quartile (<112)	0.51 (0.26–1.00)	
2 Mid quartiles (112–186)	1	
Highest quartile (>186)	0.76 (0.41–1.41)	
Milk and milk products		0.842
Lowest quartile (<417)	1.18 (0.64–2.18)	
2 Mid quartiles (417–932)	1	
Highest quartile (>932)	1.12 (0.57–2.21)	

<sup>1</sup>Energy included in the model.

<sup>2</sup>Adjusted for sex, area of birth, duration of gestation, maternal age, maternal vocational education, maternal smoking during pregnancy, number of siblings, parental asthma, parental allergic rhinitis, birth weight, mode of delivery, atopic eczema at the age of six months, pets at home, farming, contact with cowsheds during the first year of life and the duration of total breastfeeding.

<sup>3</sup>Adjusted for sex, area of birth, duration of gestation, maternal age, maternal vocational education, maternal smoking during pregnancy, number of siblings, maternal allergy, parental allergy, birth weight, mode of delivery, duration of breastfeeding, atopic eczema at the age of six months, pets at home, farming, contact with cowsheds during the first year of life and fat consumption during pregnancy.

<sup>4</sup>Industrial fat-mixes and animal fats.

<sup>5</sup>p for overall test

### 5.3 Food consumption during infancy and childhood and the risk of asthma (Study III)

The estimated median (inter-quartile range; IQR) intake of breast milk in grams per day was 860 (28–989) and 231 (0–693) at the ages of 3 and 6 months, respectively. At the age of twelve months, 13.5% of the children were breastfed and among those the intake of breast milk was 344 (170–563) grams.

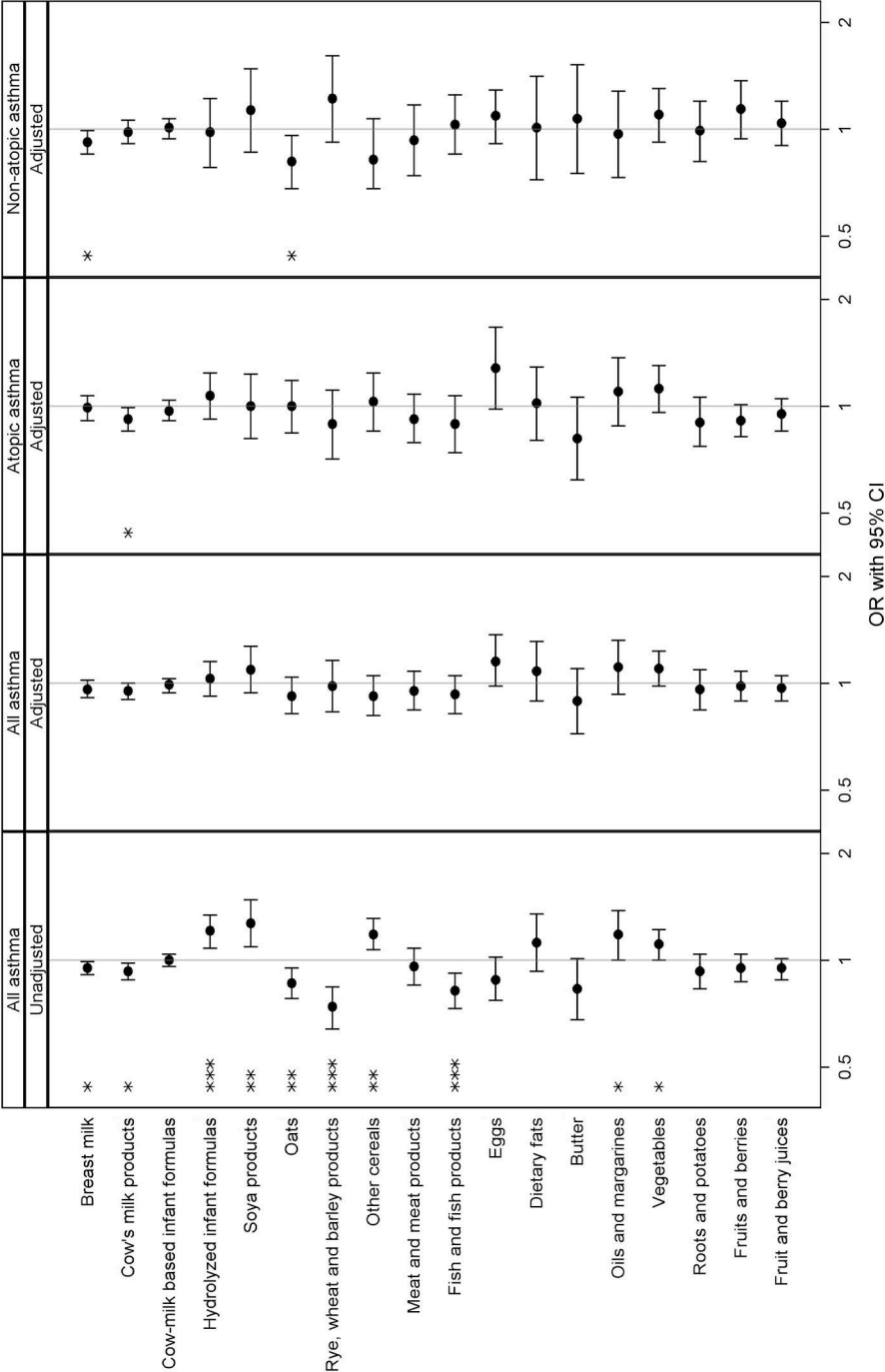
Of the background characteristics, full-term pregnancy, introduction of oats, barley, wheat and rye at the age of 5–5.5 months, fish between the ages of 6.1–8 months and egg at the age of ten months or less were associated with a decreased risk of asthma. A duration of total breastfeeding less than nine months was associated with an increased risk of asthma.

Higher consumption of breast milk, cow's milk products, oats, wheat, barley and rye products and fish and fish products was associated with a decreased risk of asthma in the unadjusted model. By contrast, higher consumption of soy-products, other cereals (rice, buckwheat, corn flour, millet) and vegetables were associated with an increased risk of asthma. After adjusting for potential confounding variables (see 4.6.1. and 4.6.2.) including CMA, higher consumption of fish and fish products was associated with a decreased risk of asthma. After further adjustment for the age at introduction of fish, the finding lost its statistical significance, suggesting that early introduction of fish may be more important than the amount consumed. It is probable that the age at introduction and amounts consumed are highly correlated, and that may have an impact on the results.

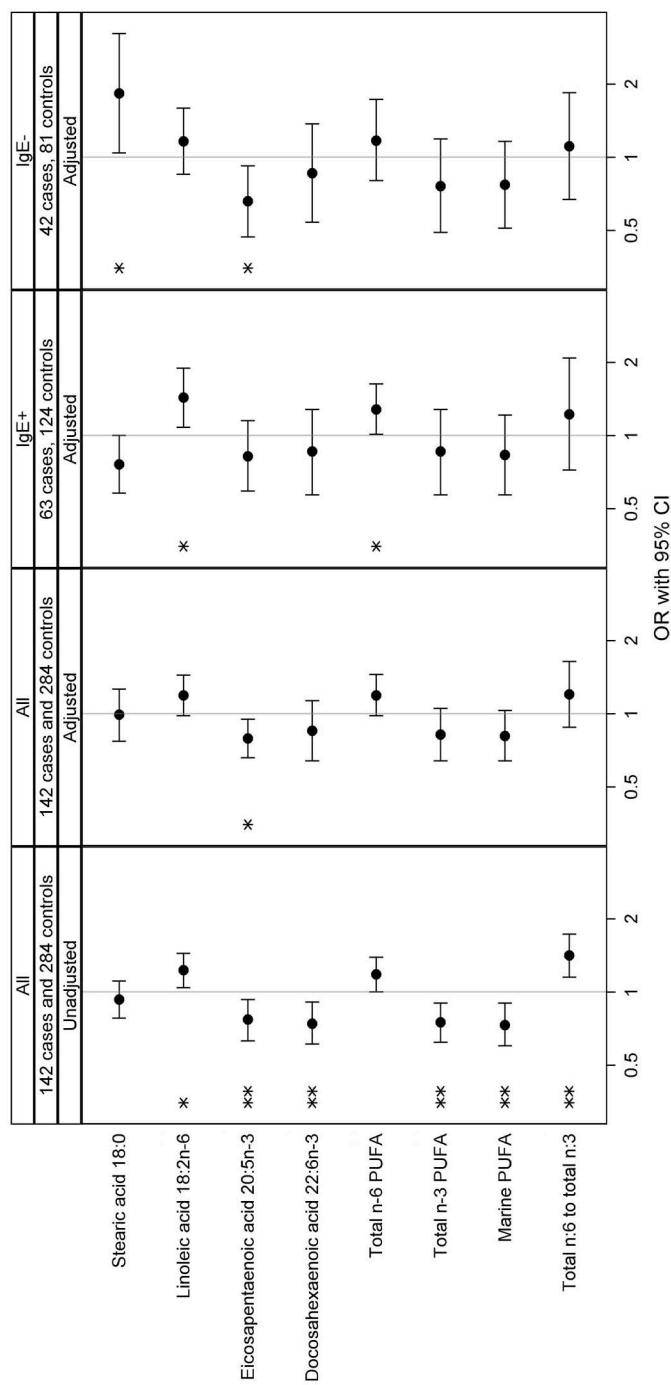
When stratifying for IgE-positivity (atopic vs. non-atopic asthma), a higher consumption of cow's milk products was inversely associated with the risk of atopic asthma. Furthermore, a higher consumption of breast milk and oats were inversely associated with the risk of non-atopic asthma.

Children with CMA consumed less cow's milk products and cow's milk-based infant formulas, as expected. In addition, they consumed less oats, wheat, barley and rye products, meat and meat products, fish and fish products, oils and margarines, roots and potatoes, fruits and berries as well as fruit and berry juices compared with those with no history of CMA. However, at the age of four years, the diet of children who had or had had CMA contained less fish and fish products and cow's milk products, but more fruits and berries, and fruit and berry juices than the diet of those children with no history of CMA.





**Figure 5.** Odds ratios (OR) (95% confidence intervals (CI)) for the risk of asthma associated with food intake during infancy and childhood analyzed longitudinally until the diagnosis of asthma in all subjects and separately in IgE-positive and IgE-negative subjects.  
\*p≤0.05 \*\*p≤0.01 \*\*\*p≤0.001 \*\*\*\*p≤0.0001



**Figure 6.** Odds ratios (95% CI) for the risk of asthma associated with the serum fatty acid composition analyzed longitudinally until the diagnosis of asthma in all study subjects and separately in IgE-positive and IgE-negative subjects. Odds ratios describe the change in risk, when serum FA composition is changed by an amount corresponding to its s.d. \*p≤0.05 \*\*p≤0.01 \*\*\*p≤0.001

## 5.4 Serum fatty acids and the risk of asthma (Study IV)

In the unadjusted model, increasing serum proportions of total n-6 PUFA and LA, and the ratio of total n-6 to n-3 PUFA, were associated with an increased risk of asthma. Conversely, increasing proportions of total n-3 PUFA, marine PUFA, EPA, CLA and palmitoleic acid were associated with a decreased risk of asthma. After adjusting for all the putative confounders, only EPA remained inversely associated with the risk of asthma. Higher proportions of LA and n-6 PUFA were associated with an increased risk of atopic asthma. Higher proportions of stearic acid and total n-6 PUFA were associated with an increased risk and that of EPA with a decreased risk of non-atopic asthma (Figure 6).

We found differences in median serum FA proportions at the age of one year between children who had CMA (n=59) compared with those who had not (n=320). In children with CMA, the serum proportions of oleic acid, LA, ALA and total n-6 PUFA were higher, and all the other serum FA proportions lower, compared with those children with no history of CMA.

## 6 Discussion

### 6.1 Summary of the findings

Dietary intake in early life, already during perinatal period, combined with a history of atopy, was associated with the risk of childhood asthma. Higher maternal intake of ALA, total n-3 PUFA and palmitic acid during pregnancy was inversely associated with the risk of asthma in the offspring, while that of AA positively associated the risk of asthma in the offspring. However, we found no associations between the main dietary sources of these FA and the risk of childhood asthma. During lactation, the associations of FA and childhood asthma could not be demonstrated, although the results were adjusted with the corresponding FA or fat intake during pregnancy.

The diagnosis of CMA in children lead to multiple dietary restrictions still evident at four years of age. Even after adjusting for CMA, higher consumption of cow's milk products was inversely associated with the risk of atopic asthma and higher consumption of breast milk and oats inversely with non-atopic asthma. Early introduction of fish was inversely associated with the risk of all asthma.

Finally, the results of this study indicate that longitudinal serum FA composition during early childhood (by the age of five years) may predict the later development of childhood asthma. The FA proportions of the children with a diagnosis of CMA differed from those without CMA, as did asthma risk. Of the 17 FA studied, after adjusting for CMA, only a higher proportion of EPA was inversely associated with a risk of asthma, particularly non-atopic asthma. Higher proportions of LA and n-6 PUFA were positively associated with the risk of atopic asthma. In addition, higher proportions of stearic acid and total n-6 PUFA were associated with an increased risk of non-atopic asthma. CMA may be both a pathway and a confounding variable, and is an important factor in studies investigating the associations between serum FA proportions and asthma. There may be changes in serum FA composition already before CMA. In addition, the diagnosis of CMA leads to a disease-related modification of diet and thus different serum FA proportions in children on a CMA elimination diet compared with children with no history of CMA.

## 6.2 Strengths and limitations of the study

### 6.2.1 Study design and endpoints

In a prospective birth cohort study, the collection of the data before the development of the disease excludes selection of the subjects according to the endpoint. In this study design, all the information needed can be collected and multiple associations of a single exposure examined (Willett 1998). It also allows measurement of the incidence of disease in a cohort. A limitation is that this design may be expensive and time-consuming. Statistical analyses of large cohorts are challenging and we have often used the knowledge and help of statisticians with our analyses. The validity of the results can also be affected by losses to follow-up (Willett 1998).

A basic feature of the nested case-control design is that the selection of controls is made independently at different failure times (i.e. the age at onset of asthma in our study) by random sampling from the non-failing subjects in the risk set (i.e. the asthma-free children at that failure time). Thus, the subject may serve as a control for more than one case, and the subject who was already a control could become a case at a later time point. This design is generally accepted and valid for assessing exposure-disease associations (Colditz *et al.* 2010).

The strength of the study is the collection of the dietary data and serum data before the development of asthma. Asthma diagnosis was assessed using a validated questionnaire (Nwaru *et al.* 2011). The age of five years was selected to exclude younger children who commonly have wheezing with viral infections indicating transient wheezing. We selected persistent asthma as our endpoint.

Food allergy diagnoses are problematic both clinically and in epidemiological studies. Diagnostic methods vary between clinics and physicians. DBPCFC is rarely performed, and easier methods like single blind and open food challenges are used instead; the occurrence of food allergies is easily overestimated based on children's symptoms. In epidemiological studies the diagnoses are based on parental reports and can thus be even more unreliable due to overreporting and reliance on memory on starting and ending times (Muraro *et al.* 2014).

The specific IgE concentrations were measured from serum samples obtained at the age of five years for the following allergens: egg, cow's milk, fish, wheat, house dust mite, cat, Timothy-grass and birch. Atopy was defined as a sensitization ( $\geq 0.35$  kU/l) to any of these allergens. Low total IgE has a strong negative predictive value (Chung *et al.* 2014). However, elevated IgE-levels do not necessarily indicate clinical symptoms of allergy. Food-related IgE-levels are higher in young children and lower when the children grow older, whereas inhalant allergen related IgE-

levels are low in young children and higher in older children. Early sensitization to inhalant allergens has been shown to be associated with later asthma and allergy in children (Csonka *et al.* 2000, Piippo-Savolainen *et al.* 2007, Ahmad Al Obaidi *et al.* 2008).

### 6.2.2 Subjects and generalizability

All age-suitable children born in the catchment areas of Oulu, Turku and Tampere University Hospitals were invited to participate in the study, thus representing the Finnish population from different geographical areas.

The study subjects belong to the DIPP cohort, which is based on a genetic risk for type 1 diabetes. The genetic risk for type 1 diabetes may limit the generalizability of the results to the general population. Type 1 diabetes is a T-helper 1 (Th1) disease, whereas asthma and allergic diseases are Th2-related diseases (Fsadni *et al.* 2012). However, common environmental and genetic factors may be related to type 1 diabetes, wheezing and atopic eczema (Fsadni *et al.* 2012). A recent study reported that type 1 diabetes in children with allergic rhinitis could prevent the development of asthma in those children (Tosca *et al.* 2013). In another study, the prevalence of allergic diseases and sensitization on children with type 1 diabetes was higher than expected (Villa-Nova *et al.* 2014). However, interactions between type 1 diabetes and asthma remains controversial (Tosca *et al.* 2009, Seiskari *et al.* 2010). The present cumulative incidence of asthma in the study population (6%) was comparable to the general population of the same age group in Finland (Kaila *et al.* 2009).

### 6.2.3 Food frequency questionnaire, three-day food records and serum samples

The food frequency questionnaire (FFQ) is the most widely used method because it is applicable to large cohorts and provides information on a wide range of foods (Willett 1998, Erkkola *et al.* 2001, Litonjua 2008). It assesses long-term food use, is easy to administer and allows repeated measurements. However, it must be validated to a particular population to ensure that the main foods are included, and the completion of the FFQ is time-consuming. The FFQ used in our study was validated and designed to reflect specifically the food use of pregnant Finnish women and the diet during the eighth month of pregnancy was investigated (Erkkola *et al.* 2001, Prasad *et al.* 2010). Mothers completed the pregnancy FFQ concerning their diet during the eighth month of pregnancy and the lactation FFQ concerning the diet during the third month of lactation retrospectively. The eighth month of pregnancy reflects the time just before maternity leave in Finland. The design is retrospective because of the recruitment of study subjects after birth. The third month of lactation

was chosen because most mothers are still breastfeeding when the child is three months old (Erkkola *et al.* 2001, Prasad *et al.* 2010).

FFQs usually overestimate the intake of foods and nutrients (Erkkola *et al.* 2001, Burrows *et al.* 2010, Jackson *et al.* 2011). This is taken into account by energy adjusting the food and nutrient intakes using the Willet's residual method (Willet 1998). The FFQ was validated and found suitable for pregnant Finnish women (Erkkola *et al.* 2001). The strength of the present study is that lactation data is adjusted for pregnancy data, thus taking into account the maternal diet during pregnancy when analyzing lactation data. The FFQ was not validated for lactating women and this may cause biased results. Web-based instruments are currently replacing FFQ in dietary assessments (Boeing *et al.* 2013).

Three-day food records are the gold standard of nutritional methods (Burrows *et al.* 2011). In the food record method, the subjects prospectively document the foods they have consumed to avoid dependence of memory (Litonjua 2008). However, parents may alter the diet of their children when keeping food records to provide a healthier picture of the dietary habits of the family, or to make it easier to complete the records. Underreporting in food records is common (Burrows *et al.* 2011). However, parents and day care personnel were observed not to alter the normal eating habits. The day-to-day variation in food consumption increases by age and is higher in girls than boys. In addition, the record should include one weekend day, because diet is different between weekdays and weekend days (Burrows *et al.* 2011, Erkkola *et al.* 2011). Our food records were validated against biomarkers such as serum FA and vitamin E concentrations (Uusitalo *et al.* 2013).

Food composition databases (Fineli in Finland) provide reliable information on amounts of energy and various nutrients in foods, and they are usually country-specific (Uusitalo *et al.* 2012). Nutrient values in the food composition database must be updated frequently and new foods and recipes must be added promptly to get accurate information for example for study purposes. However, the nutrient content can vary between different varieties of same foods, thus affecting the accuracy of the nutrient, for example FA, calculations by food composition databases (Uusitalo *et al.* 2012).

Tissue and serum FA have been used as biomarkers of dietary intake. Biomarkers are used in epidemiological studies to validate information from FFQ and also indicate the status of an individual with regard to the nutrient of interest (Litonjua 2008). There are principal validated biochemical markers suitable for using in epidemiological studies for the following FA (in plasma and/or adipose tissue): palmitic acid, oleic acid, LA, trans FA, EPA and DHA (Willett 1998). Serum cholesteryl ester is considered as most suitable serum fraction to reflect short-term

dietary intake whereas adipose tissue FA are biomarkers for long-term dietary intake (Baylin *et al.* 2006). However, these biomarkers are invasive, expensive and laborious, thus not widely used in epidemiological studies.

In a systematic review of the validity of dietary methods in children compared with the gold standard method of doubly labeled water, over-reporting was associated with 24-hour diet recalls and FFQs while underreporting was often associated with food records (Burrows *et al.* 2010). In four to eleven-year old children, the 24-hour recall provided by the parents over three days including weekdays and the weekend was the most accurate method. In children aged six months to four years, a food record was the recommended method, while in adolescents over sixteen years, a diet history provided the most accurate information on diet (Burrows *et al.* 2010).

### 6.3 Comparison of results with earlier studies

#### Study I

Our study indicated that a higher maternal intake of ALA, total n-3 PUFA and palmitic acid during pregnancy were inversely associated with the risk of asthma in the offspring, while that of AA was positively associated with the risk of asthma in the offspring. However, we found no associations between the main dietary sources of those FA and childhood asthma risk.

In line with our results, maternal intake of fish during pregnancy has been associated with a reduced risk of asthma in children in earlier epidemiological studies (Kremmyda *et al.* 2011). A Japanese study also found that a higher maternal intake of ALA during pregnancy may reduce the risk of infantile wheeze (Miyake *et al.* 2009). The finding that a low intake of AA decreases the risk of childhood asthma supports the original lipid hypothesis. (Black *et al.* 1997). The protective effect of SAFA, especially palmitic acid, is not supported by earlier pregnancy studies. SAFA has not shown anti-inflammatory properties. By contrast, SAFA may contribute to rising propensity for chronic low-grade inflammation, which is a risk factor for later chronic diseases (Amarasekera *et al.* 2013). The consumption of SAFA may be a proxy of an unhealthy dietary pattern, since dietary monounsaturated FA may be exchanged for dietary SAFA (Yacoob 2002). The finding of a protective effect of SAFA may somehow be related to CMA and a disease-related modification of the diet.



## Study II

During lactation, the associations between FA and childhood asthma could not be demonstrated, even when the results were adjusted with the corresponding FA or fat intake during pregnancy. Similarly to our findings, in a systematic review, n-3 PUFA supplementation during lactation did not prevent asthma, food allergy or atopy (Klemens *et al.* 2011). By contrast, a Swedish cohort study reported that maternal fish oil supplementation during pregnancy and lactation was associated with a lower cumulative incidence of allergic sensitization and IgE-mediated allergic diseases in children up to two years of age. A higher proportion of n-3 PUFA in maternal and infant phospholipids due to fish oil supplementation also resulted in a lower frequency and milder symptoms of allergic diseases up to two years of age (Furuhjelm *et al.* 2011). Nutrients can modify immune and metabolic programming during fetal and early postnatal periods (Amarasekera *et al.* 2013, Palmer *et al.* 2014). Intrauterine time has been suggested as the most critical time in programming. Our study also suggests that diet during pregnancy is more important in programming compared to lactation period. The inconsistent results may also be due to differences in the amount of foods or FA consumed, in size of the study populations or in type of the study.

## Study III

When studying the relationship of food consumption to outcomes such as asthma, previous dietary history and being atopic or not requires careful consideration. CMA resulted in dietary restrictions up to four years of age. Taken this into account, higher consumption of cow's milk products was inversely associated with atopic asthma. Earlier studies have reported associations between higher consumption of fruits, vegetables, nuts, wholegrain cereals and whole-fat dairy products during childhood and a decreased risk of asthma (Dunder *et al.* 2001, Barros *et al.* 2011, Willers *et al.* 2011, Saadeh *et al.* 2013). Regular milk intake has been associated with a decreased risk of asthma and allergic diseases (Suarez-Varela *et al.* 2010). A prospective birth cohort study from the Netherlands reported that frequent intake of high-fat milk among pre-school children was inversely associated with the risk of asthma and wheezing at the age of three years (Wijga *et al.* 2003). Early life consumption of unpasteurized cow's milk has been associated with a reduced risk of allergies and asthma in children (von Mutius 2012, Sozanska *et al.* 2013). Both the microbial contamination of milk and the whey fraction of unprocessed cow's milk have been thought to be beneficial against atopic diseases and the development of tolerance.

Higher consumption of oats was inversely associated with the risk of non-atopic asthma. The early introduction of oats has been associated with a lower risk of

asthma in a smaller cohort in the DIPP Study (Virtanen *et al.* 2010). Oats may have immune-modulatory and anti-inflammatory properties (Davis *et al.* 2003). In a prospective birth cohort study from the Netherlands, daily consumption of brown bread was inversely associated with the risk of asthma (Wijga *et al.* 2003).

The present finding that a higher consumption of breastmilk is inversely associated with asthma risk is novel. A longer duration of total breastfeeding has been shown to be associated with non-atopic asthma (Nwaru *et al.* 2013). In addition, breastfed children are proposed to have better lung function (von Mutius 2012). The etiology of asthma is complex with different phenotypes, and breastfeeding may variously affect on those phenotypes. Genetic variations in fatty acid desaturases, FADS1 and FADS2, have been shown to influence maternal plasma and breast milk FA during pregnancy and lactation (Xie *et al.* 2008). FADS gene clusters may also influence the risk of developing atopic disease (Standl *et al.* 2011, Standl *et al.* 2012).

#### Study IV

A higher serum proportion of EPA was associated with a decreased risk of asthma, especially non-atopic asthma, when CMA was considered as a putative confounder. Higher proportions of LA and total n-6 PUFA were associated with an increased risk of atopic asthma. Higher proportions of stearic acid and total n-6 PUFA were associated with an increased risk of non-atopic asthma.

In keeping with the present study, a case-control study among Korean pre-schoolers showed that total red blood cell n-3 PUFA were lower in atopic children compared with healthy controls (Hwang *et al.* 2007). In addition, in an Australian randomized controlled trial, a higher proportion of n-3 PUFA in plasma in eighteen-month-old children was associated with reduced wheezing, nocturnal cough and the use of bronchodilators (Mirshahi *et al.* 2004). Furthermore, high plasma concentrations of EPA and DHA in children up to age of two years have been implicated in decreasing the risk of IgE-mediated allergic diseases and reducing the severity of the allergic phenotype (Furuhjelm *et al.* 2011).

Increasing proportions of LA and total n-6 PUFA were associated with an increased risk of atopic asthma. These findings agree with the results of a German case-control study, which reported that children with atopic asthma had higher levels of LA in their plasma than the healthy controls (Leichsering *et al.* 1995). Kankaanpää *et al.* observed higher levels of n-6 PUFA in serum phospholipids of atopic infants compared with the controls (Kankaanpää *et al.* 2001). In another Finnish study, similar to our results, serum proportions of LA and n-6 PUFA were higher in children with atopic eczema compared with those with non-atopic eczema (Laitinen *et al.* 2006).

It has been suggested that FA metabolism is disturbed in atopic diseases (Focke *et al.* 2005, Sala-Vila *et al.* 2008). One proposed mechanism is the impaired function of the enzyme delta-6-desaturase, which decreases the synthesis of long chain PUFA from their precursors. Dysregulation of PUFA metabolism is associated with an increased IgE production and atopy (Sala-Vila *et al.* 2008). However, this proposition was not confirmed in a Swedish study of school-aged children, which suggested that the changes in PUFA metabolism are associated with allergic diseases themselves (Yu *et al.* 1998).

There is evidence that, in addition to the diet, the PUFA composition in plasma and tissues is modulated by genetic variation in FA desaturase (FADS) gene clusters (Glaser *et al.* 2011). Several studies have shown strong associations between the FADS gene cluster and FA levels in serum phospholipids, plasma and adipose tissue samples, erythrocyte cell membranes, breast milk and red blood cell lipids (Standl *et al.* 2011). Genetic variants of FADS have been shown to influence blood lipid FA status during pregnancy and lactation (Xie *et al.* 2008), and also the incidence of allergic rhinitis and atopic eczema (Schaeffer *et al.* 2006). Also, the association between dietary fat intake and allergic diseases in childhood may be modulated by genetic variation in FADS (Standl *et al.* 2011, Standl *et al.* 2012). This genetic variation could be one reason for conflicting results of the associations between FA and atopic diseases.

Reverse causality needs to be taken into account in studies relying on information from the parents. Another important reason for discordant results of the earlier studies may be that CMA and the CMA-related elimination diet affecting the serum FA profiles have not been taken into account in most of these studies. Earlier studies investigating the associations between food intake and allergic diseases and asthma have taken the disease-related modification of the diet into account in the analyses by excluding children with food allergies (Wijga *et al.* 2003, Kim *et al.* 2005, Rosenlund *et al.* 2011) or children with symptoms of wheeze and eczema during the first year of life (Magnusson *et al.* 2013), or used doctor-diagnosed food allergy during the first year of life as a confounding factor (Alm *et al.* 2011, Goksor *et al.* 2011). To our knowledge, the previous surveys analyzing the relationship between serum FA profiles and asthma were not controlled for food allergies. Changes in serum FA composition may predispose to CMA and consequently to asthma because these diseases are on the same spectrum. A CMA diagnosis results in the elimination of milk, dairy products and many other foods from the diet, and thus further changes in serum FA profiles. These changes may modify the immunological reactions of the child already in early life. Clearly, as seen in the current study, the FA proportions of children with CMA differed from those with no history of CMA, as did the asthma risk of the groups. Hence, after adjusting for CMA, only EPA remained

inversely associated with the risk of asthma, while other associations found prior to CMA adjustment lost their statistical significance.

## 7 Conclusions and future implications

This study provides evidence that early life diet is associated with a later risk of childhood asthma. Maternal ALA, total n-3 PUFA and palmitic acid intake during pregnancy may decrease the risk of asthma in the offspring, while AA intake may increase the risk of asthma in the offspring. During lactation, associations between FA or fat intake and childhood asthma could not be demonstrated even when adjusting for the corresponding FA or fat intake during pregnancy.

Diagnosis of CMA resulted in dietary restrictions up to four years of age. When studying the relationship of food consumption to outcomes such as asthma, previous dietary history and being atopic or not should be carefully considered. Cow's milk restriction due to CMA significantly affects and modifies the association between food consumption and childhood asthma risk. Higher consumption of breast milk was inversely associated with the risk of asthma. To our knowledge, no earlier studies have investigated the impact of the amount of breast milk consumed per day on the risk of childhood asthma.

CMA confounded the associations between serum FA and asthma. Changes in serum FA composition may predispose to CMA and consequently to asthma. After adjusting for CMA, only a higher serum proportion of EPA was inversely associated with the risk of asthma. To our knowledge, no previous studies have prospectively examined the associations between repeated serum FA proportions and the onset of asthma in children. In addition, the previous studies did not control for food allergies.

A CMA diagnosis results in the elimination of milk, dairy products and many other foods from the diet, and thus further changes in serum FA profiles. These changes may modify the immunological reactions of the child in early life. Elimination diets because of food allergies should be taken into account in further studies. Future studies investigating the role of diet on asthma and allergic diseases should focus on antenatal period when it is possible to modify the critical time in developmental programming. Also, it would be important to study the associations between serum FA proportions already during the first year of life and childhood asthma and other allergic diseases. The role of FADS should also be taken into account in further analyses.

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Sipoo, November 2014

Mirka Lumia



## APPENDIX

### The process of the literature review

The literature review was based on searches of the PubMed and Ovid MEDLINE®, Ovid MEDLINE® Daily Update and Ovid MEDLINE® In-Process & Other Non-Indexed Citations databases:

#### PubMed

(ASTHMA) and (n-6 fatty acids) 97

(ASTHMA) and (n-3 fatty acids) 236

(ASTHMA) and (dietary fatty acids) 293

((("Fatty Acids, Omega-6"[MeSH Major Topic]) OR ("Fatty Acids, Omega-3"[MeSH Major Topic])) AND ("Asthma"[MeSH Major Topic])) 78

((("Fatty Acids, Omega-6"[MeSH ]) OR ("Fatty Acids, Omega-3"[MeSH ])) AND ("Asthma"[MeSH Major Topic]) ) NOT ((("Fatty Acids, Omega-6"[MeSH Major Topic]) OR ("Fatty Acids, Omega-3"[MeSH Major Topic])) AND ("Asthma"[MeSH Major Topic])) 37

(Fatty Acids, Omega-6 OR Fatty Acids, Omega-3) AND Asthma 250

((("Fatty Acids, Omega-6"[MeSH]) OR ("Fatty Acids, Omega-3"[MeSH])) AND ("Asthma"[MeSH Major Topic])) NOT ((("Fatty Acids, Omega-6"[MeSH Major Topic]) OR ("Fatty Acids, Omega-3"[MeSH Major Topic])) AND ("Asthma"[MeSH Major Topic])) AND All Child 15

(((((("Fatty Acids, Omega-3/blood"[MAJR]) OR "Fatty Acids, Omega-6/blood"[MAJR]) OR "Palmitic Acid/blood"[MeSH Terms]) OR "Oleic Acid/blood"[MeSH Terms]) OR "Cholesterol Esters/blood"[MAJR]) OR "Arachidonic Acid/blood"[MeSH Terms]) AND Asthma 20

diet and asthma and child 579

food consumption and asthma and child 165

((((serum OR blood)) AND (fat OR fatty acid)) AND child) AND asthma 240

diet AND "fatty acids" AND asthma 162

((("Fatty Acids, Omega-6"[MeSH]) OR ("Fatty Acids, Omega-3"[MeSH])) AND ("Asthma"[MeSH Major Topic])) NOT (("Fatty Acids, Omega-6"[MeSH Major Topic]) OR ("Fatty Acids, Omega-3"[MeSH Major Topic])) AND ("Asthma"[MeSH Major Topic]) 37

### **Ovid MEDLINE®, Ovid MEDLINE® Daily Update and Ovid MEDLINE® In-Process & Other Non-Indexed Citations databases**

#### **Study I**

exp Dietary Fats, Unsaturated/ exp Asthma/ exp Fatty Acids/ exp Pregnancy/ "fatty acids".ti,ab./ (prenatal or pre-natal or antenatal or ante-natal or pregnan\*).ti,ab. / asthma\*.ti,ab. 88

#### **Study II**

exp Dietary Fats, Unsaturated/ exp Fatty Acids/"fatty acids".ti,ab. / exp Asthma/ asthma\*.ti,ab. / exp Breast Feeding/ exp Lactation/ exp Milk, Human/("breast feeding" or breastfeeding).ti,ab. 52

#### **Study III**

exp Child/ exp Diet/ exp Asthma/ exp Risk Factors/ 52

#### **Study IV**

exp Serum/ exp Fatty Acids/ exp Dietary Fats, Unsaturated/ exp Asthma/"fatty acids".ti,ab./ asthma\*.ti,ab./ serum.ti,ab. 285

All the references were checked and those suitable are displayed in Tables 2 and 3.

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